UNIVERSITÀ DEGLI STUDI DI PARMA
DIPARTIMENTO DI FARMACIA
Dottorato di Ricerca in Farmacologia e Tossicologia Sperimentali
XXVIII Ciclo

Nutraceutical Approach to the Modulation of Cholesterol Metabolism: in Vitro and in Vivo Studies

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Triennio Accademico 2013-2015
Quando corro tutti i pensieri volano via.
Superare gli altri è avere la forza, superare se stessi è essere forti
Confucio
Abstract

In recent years, new ways to treat cardiovascular diseases is spreading, aimed to support the medical therapy or replace it. Indeed abandonment of therapies is the major public health problem of western world. Several causes induce a reduced adherence to the therapy, as the complexity of treatments and the side effects of administered drugs, especially for chronic diseases. Consequently, a reduction of side effects leads to an improvement of living condition of patients. In order to avoid the abandonments of therapies, it may be used a nutraceutical approach supporting pharmacological drugs. Nutraceutical therapies include molecular complexes and microorganisms, which come from plants, milk, and functional foods. The general aim of this study is to investigate the cholesterol lowering activity of a nutraceutical compound and a specific bacterial strain in animal models with high plasma cholesterol levels. In addition, the effects of nutraceutical treatment on physiological mechanisms which counteract the creation of atherosclerotic plaque such as the cholesterol efflux from foam cells of atheroma, or the cholesterol uptake from intestine, has been analysed. The present thesis is divided in two parts.

In the first part, we investigated the capability of bifidobacteria to reduce cholesterol levels in growth media, demonstrating a high assimilation of this molecule by particular bifidobacterial strains, as Bifidobacterium bifidum PRL2010. The transcriptomic analysis of Bb PRL2010 cultivated in the presence of cholesterol revealed a significantly increased transcription level of genes encoding putative transporters and reductases, indicative of specific mechanisms for cholesterol assimilation as well as cholesterol conversion to coprostanol. Cholesterol lowering activity of B. bifidum PRL2010 cells was further evaluated by means of an in vivo murine model, showing that the faecal microbiota of mice is modified toward those bacteria involved in the metabolism of cholesterol.

In the second part of research project, we investigated the activity of a nutraceutical compound patent protected called “Ola”, on the cholesterol efflux of hamsters treated with the nutraceutical compound. Cholesterol efflux is the first step of a physiological mechanism, the Reverse Cholesterol Efflux (RCT), which allows the cholesterol elimination from atherosclerotic plaques, through interaction among
HDL, which are present in the blood circulation, and specific transporters of foam cells, as ABCA1/G1 and SR-BI. Successively, these lipoproteins release the cholesterol to hepatic cells, where it is metabolized and excreted via faeces. In order to evaluate the activity of Ola treatment of lipid profile of hamsters, in vitro analysis occurred, which have shown mainly an increased cholesterol efflux in cells expressing the transporter ABCA1, compared with the control group.

These two studies show as the nutraceutical approach may be an important way against hyperlipidemic condition and atherosclerosis. As literature suggests, the effects of nutraceutical compounds against atherosclerosis and other chronic disease, led to use them in order to support pharmacological therapies or in some cases replace them.
Abstract

Negli ultimi anni, si sono diffusi nuove strategie per il trattamento delle malattie cardiovascolari, che possano supportare una terapia medica, o in alcuni casi, sostituirla. Infatti, l’abbandono delle terapie è il più importante problema di salute pubblica del mondo occidentale, soprattutto per le malattie croniche. Ciò è dovuto alla complessità delle terapie farmacologiche e ai numerosi e in alcuni casi gravi effetti collaterali dei farmaci somministrati. Di conseguenza, una riduzione di questi effetti migliorerebbe le condizioni di vita del paziente e quindi diminuirebbe il rischio di abbandono della terapia. Per ottenere ciò, è possibile affiancare al trattamento farmacologico una terapia nutraceutica, consistente nella somministrazione di complessi molecolari o microorganismi, provenienti da piante, latte o cibi funzionali.

Lo scopo generale di questo studio è indagare le attività ipolipidemizzanti di un composto nutraceutico e di un ceppo batterio specifico nel modello animale che presenta elevati alti livelli plasmatici di colesterolo. Inoltre, sono stati analizzati gli effetti del trattamento nutraceutico sui meccanismi fisiologici che contrastano la creazione della placca aterosclerotica come l’efflusso di colesterolo dalle “foam cells” presenti nell’ateroma, o la riduzione dell’assorbimento intestinale di colesterolo. La presente tesi è divisa in due parti.

Nella prima parte, abbiamo analizzato la capacità dei Bifidobacteria di ridurre i livelli di colesterolo nel medium di crescita. Dall’analisi, si è osservato che vari ceppi del genere Bifidobacteria presentano un’ampia capacità di assimilazione del colesterolo all’interno della cellula batterica, in particolare il Bifidobacterium bifidum PRL2010. Le analisi di trascrittomica del Bb PRL2010 incubato in presenza di colesterolo, hanno rivelato un significativo aumento dei livelli di trascrizione di geni codificanti trasportatori e riduttasi, responsabili del meccanismo di accumulo all’interno della cellula batterica e della conversione del colesterolo in coprostanolo. L’attività ipolipidemizzante del Bb PRL2010 è stata poi valutata nel modello murino, mostrando la modificazione del microbiota dei topi trattati dopo somministrazione del batterio in questione.
Nella seconda parte del progetto di ricerca, abbiamo indagato sugli effetti di un composto coperto da brevetto, chiamato “Ola”, sull’efflusso di colesterolo di criceti trattati con questo composto nutraceutico. L’efflusso di colesterolo è il primo step del meccanismo fisiologico noto come Trasporto Inverso del Colesterolo, che consente l’eliminazione del colesterolo dalle placche aterosclerotiche, attraverso l’interazione fra le HDL, presenti nella circolazione sanguigna, e specifici trasportatori delle foam cells, come ABCA1/G1 e SR-BI. In seguito, le lipoproteine rilasciano il colesterolo alle cellule epatiche, dove è metabolizzato ed escreto attraverso le feci. Per valutare l’effetto dell’Ola sul profilo lipidico dei criceti, sono state condotte analisi in vitro. I risultati mostrano un aumento dell’efflusso di colesterolo in cellule che esprimono il trasportatore ABCA1, comparato con il gruppo controllo.

Questi due studi mostrano come l’approccio nutraceutico può essere un importante modo per contrastare l’aterosclerosi. Come mostrato in letteratura, gli effetti dei composti nutraceutici sull’aterosclerosi e su altre malattie croniche, hanno portato a un ampio uso come supporto alle terapie farmacologiche, ed in alcuni casi hanno rimpiazzato la terapia farmacologica stessa.
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Introduction
1. Atherosclerosis

Cardiovascular diseases (CVD) are one of the major causes of death in western world and atherosclerosis is the main risk factor for CVD. Atherosclerosis is a progressive, multifactorial and chronic disease, which affects arterial vessels, manifested by the development of lesions within the arterial wall. It is originated from an inflammatory process that leads to development of atherosclerotic plaque. The atheroma is characterized by accumulation of macrophages engorged with cholesterol, called foam cells and fibrous elements. Rupture of vulnerable plaque can occlude the vessels and can lead to ischemia of the heart, brain, stroke and myocardial infarction (Santos-Gallego 2011).

The specific causes of endothelial dysfunction are not completely understood. It has been believed for many years that atherosclerosis was a result of lipids and cholesterol accumulation in arterial walls (Ross 1993). Currently, epidemiological studies have identified at least two different classes of risk factors, called constitutional risk factors (age, genes, predisposition of high blood pressure and diabetes) and behavioural (smoking, physical inactivity, unhealthy diet). The most important risk factors among those mentioned above are mutations of genes, physical inactivity and unhealthy diet.

The mutations of genes, involved in angiogenesis and blood pressure control, may increase atherosclerosis risk and heart diseases. Other etiological causes include toxins, homocysteine and even infectious agents. In addition, inflammatory cytokines secreted by leucocytes (e.g. tumor necrosis factor, or TNF-α), can also stimulate the atherogenesis. Major cause of atherosclerosis is unhealthy diet, which induces dyslipidaemia, thus abnormal values of lipids in blood (Lusis 2000).

1.1 Atherogenesis and Morphology of Atherosclerotic Plaque

Three different layers compose vessel wall: tunica intima, media and adventitia.

- The intima consists of few smooth muscle cells (SMCs) and a monolayer of endothelial cells (EC) that, with tight junctions, work as a semipermeable
barrier. Also it contributes to the synthesis of extracellular matrix (ECM) proteins, which are involved into the inflammation and vascular tone regulation.

- The media is composed by numerous layers of SMC, bordered by elastic lamina, intercalated between basal laminae and extracellular matrix. The SMCs contract, under stimulation of sympathetic nervous system, so as to allow blood pressure control.

- An external elastic lamina separates the media from the adventitia. It consists mainly of fibroblasts, mast cells, lymphatic vessels, nerves and fibres of collagen, thus providing stability to vessels (Barton 2007).

The lesions of atherosclerosis occur principally in large and medium-sized elastic and muscular arteries (aorta, carotid, coronary and iliac arteries). Due to these plaques, blood flowing decreases into the vessels, reducing oxygen and nutrient supplies to tissues. This may lead to heart and brain ischemia.

Atheroma is a vessel lesion composed of a lipid core (mainly esterified cholesterol), of cellular debris and macrophages covered by a firm fibrous cap composed of smooth muscle cells, cholesterol engorged-macrophages, collagen, elastin and proteoglycans.

During atherogenesis, a series of changes occur into the arterial wall. It is generally accepted that atherosclerosis is an inflammatory disease which can be explained as a “response-to-an-injury” (Ross 1993). Indeed lipids, free radicals, hemodynamic turbulences and hypertension factors influence the secretory activity of endothelial cells, which increases the expression of leukocyte adhesion molecules as Vascular Cell Adhesion Molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), P-selectin, E-selectin and chemotactic proteins as monocyte chemotactic protein-1 (MCP-1) (Dong 1998) (figure 1). In addition, proliferation and differentiation of macrophages, both important steps of atherogenesis, are stimulated by growth factors as macrophage colony-stimulating factor (M-CSF) (Smith 1995). Also some enzymes may be pro-inflammatory agents, as the Myeloperoxidase (MPO). This is a lysosomal protein stored into the granules of neutrophils; it is released into the extracellular space during inflammation. It catalyses the chlorination and nitration of tyrosyl residues of apolipoprotein A-I (apoA-I) (Kameda 2015); as well,
the enzyme *Group II secretory Phospholipase A2* lead to LDL oxidation, as in vivo experiments on mice suggest (Ivandic 1999);

Endothelial changes lead to increase of apoB rich lipoprotein uptake, as Low Density Lipoproteins (LDL) and Very Low Density Lipoprotein (VLDL), by monocyte-derived macrophages located into the intima (Cybulsky 2010). Lipoproteins turn into oxidized LDL, after interactions with reactive oxygen species (ROS) secreted by monocytes and pro-inflammatory agents, inducing the production of chemokines and adhesion molecules mentioned above, thus contributing to the dysfunction of endothelium (Glass 2001). Within the intima, the oxLDLs do not interact with the ordinary endocytic LDL receptor, but they are recognized by macrophage scavenger receptors, which are not able to limit the internalization of lipids. This leads to lipid engulfment, which triggers fatty streaks formation (Kzhyskowska 2012).

Macrophage containing oxLDL are called “foam cell”, which represents the characteristic atherosclerosis feature and significantly contributes to the development of early sub-endothelial lesions, called fatty streak. Consequently, macrophage lipid accumulation represents a key mechanism in the development of atherosclerosis and molecular processes addressed to reduce foam cells formation might assume an athero-protective function (Walker 1986). If lipid deposition within the arterial wall
persists, inducing a fatty streak increase, pro-inflammatory response continues and smooth muscle cells (SMCs) migrate from the media layer and start to proliferate, mediated by many factors as Platelet-Derived Growth Factor (PFGF). During this stage, SMCs produce extracellular matrix rich in collagen, elastin and proteoglycan, which contributes to the creation of fibrous cap described above. This cap typically overlies the growing atheroma, mostly composed by collection of foam cells, some of which die (apoptosis) and release lipids and cholesterol into the plaque (figure 1). The inefficient clearance of dead cells, a process known as efferocytosis, may promote the accumulation of cellular debris, extracellular matrix and extracellular lipids, forming a lipid-rich pool called the necrotic core of the plaque (Libby 2011).

The progression of the inflammatory process allows the lesion to evolve into intermediate lesion supported by angiogenesis (Lusis 2000). Successively, in advance stages, the calcification process occurs into the intima, among vascular smooth muscle cells and the surface of atheroma plaques. The storage of calcium crystal in the plaque decrease the elasticity of vessels (King 2008). This may causes bleeding, wear of luminal surface and thrombus, which is the most serious intimal lesion. The rupture of atheroma releases thrombi in the blood, mostly composed of red and white blood cells and fibrin. Those are pro-thrombotic components that active the platelets. They are responsible of the creation of other thrombi, which partially or totally occlude vessels, decreasing hematic amount to tissues, very dangerous for heart and brain (Figure 2) (Libby 2005).
1.2 Lipoproteins and Lipid Metabolism

Lipids, cholesterol and triglycerides are non-polar molecules; therefore they form a different phase from aqueous of blood. To address this problem, lipids and cholesterol are carried into the circulation system by lipoproteins, which are hydrophobic and amphipathic structures.

All different lipoproteins have the same structure micelle-like. The core of the micelle is composed by esterified cholesterol and triglycerides, enveloped by an amphipathic monolayer of non-esterified cholesterol, phospholipids and apolipoproteins. These proteins have structural functions and in addition act as ligands with cellular receptors and transporters involved in lipid metabolism (Wasan 2008).

Lipoproteins are divided in five classes, according to differences of density, obtained with density-gravity ultracentrifugation and proteins composition (figure 3):
- Chylomicrons, which present the lowest density and the biggest size and are synthetized in the intestine from triacylglycerols, lipids and apolipoprotein B48;
- Very low density lipoproteins (VLDL) mostly composed by triacylglycerols, free and esterified cholesterol. Their main function is releases triacylglycerols from liver to peripheral tissues;
- Intermediate Density Lipoproteins (IDL) derived from VLDL, they have lost most of triglycerides but retain cholesteryl esters;
- Low density lipoproteins (LDL) mostly composed by cholesterol and cholesteryl esters, they are the main transporter of cholesterol in humans (Mahley 2006),
- High density lipoprotein (HDL) are the smallest lipoproteins and transport cholesterol from peripheral tissue to the liver.

Lipoproteins are spherical or discoidal particles with diameters ranging from 35 Å to 6 μm. As showed in figure 3, there are two classification systems for lipoproteins. The former is based on the density of the particles; the latter is based on electrophoretic mobility. The density nomenclature includes VLDL (0.94 < δ < 1.006 g per mL), LDL (1.006 < δ < 1.063 g per mL), HDL (1.063 < δ < 1.21 g per mL) and the largest of the particles, chylomicrons (δ < 0.94 g per mL). Lipoproteins have additionally been classified by their electrophoretic mobility as α (HDL), pre-β (VLDL) and β (LDL). This nomenclature does not include the chylomicrons, because they do not exhibit electrophoretic mobility (Vance 2002).

Chylomicrons are formed in enterocytes through the packaging of nascent triacylglycerols (80-88%) with other lipids and apolipoprotein B48 (ApoB48), which represents less than 3% w/w. These particles are then secreted from the enterocyte into the mesenteric lymph. Each chylomicron contains ApoB48, a truncated form of apolipoprotein ApoB (Powell 1987). Moreover chylomicrons contain ApoAI, which is immediately transferred to HDL, when chylomicrons are in the blood stream.

The next class of lipoprotein particles is very low density lipoproteins (VLDLs). Its main function is the removal of excess triacylglycerols from hepatocytes for use by peripheral tissues. Similar to chylomicrons, they contain ApoB and they are primarily composed of triacylglycerols (around 45–50% w/w), but also contain free
and esterified cholesterol. These cholesterol and glycerols are released through the lipolytic actions of lipoprotein and hepatic lipase, which releases almost 90% of the triacylglycerol content, turning it in free fatty acids (Tuin 2015). The VLDL remnants are partially cleared by hepatic receptors, but the majority (70%) remains into the plasma compartment for subsequent conversion to LDL through the continued action of cholesteryl ester transfer protein (CETP) and the loss of ApoE. CETP mediates the transfer of cholesteryl esters from ApoAI-containing particles to ApoB-containing particles, and opposite direction for the triacylglycerols (Morton 2007). The removal of triacylglycerol content by lipoprotein lipase converts VLDL particles into smaller, more stable, low density lipoproteins (LDLs). LDL particles are the primary transport mechanism for the delivery of cholesterol to peripheral tissues, almost 70–80% of the circulating cholesterol in humans. Unlike chylomicrons and VLDL particles, LDL particles (range size 18 to 25 nm) are composed mostly of cholesterol and cholesteryl esters and contain a single molecule of ApoB100 (Vance 2002).

1.3 Role of High Density Lipoproteins (HDL) in Lipid Metabolism

High density lipoproteins (HDLs) are the smallest lipoprotein particles (12 nm diameter) with the highest protein content. The physiological role of HDL heterogeneous population is the transport of unesterified cholesterol from the peripheral tissues to the liver.
The prevalent characteristic of HDLs subclasses is the relatively high apolipoprotein content (35–56% w/w), composed of ApoAI and ApoAII. The cholesterol content is esterified by the circulating enzyme *lecithin–cholesterol acyltransferase* (LCAT) and integrated into the HDL core (Rader 2006). According to different structural properties, HDL can be divided into several classes. First class is based on HDL shape, in particular discoidal or spherical shape. Second mode consists in HDL classification by density of particles. Two different lipoprotein family obtained are HDL2 (1.063-1.1125 g/ml) and HDL3 (1.125-1.121 g/ml) (Havel 1955). Internally each class can be divided into HDL2b, HDL2a, HDL3a, HDL3b, and HDL3c by gradient gel electrophoresis.

Another type of HDL classification is based on electrophoretic mobility. This method makes possible to determine the α-HDL class, which presents a high negative surface charge, and the preβ-HDL which mainly contains ApoAI and phospholipids (Rothblat 2010). Finally, using immune-affinity methods HDL can be divided into two classes based on apolipoprotein component: LpA-I, LpA-II and LpA-I which contains only ApoAI (Figure 3) (Scanu 2008).

Epidemiological evidence indicates that high levels of HDL cholesterol (HDL-C) are associated with a decreased risk of cardiovascular disease (Shin 2014). HDL particles are involved in an important process for cholesterol modulation: the reverse cholesterol transport (RCT) pathway, which implicates the transport of excess cholesterol from peripheral tissues and atherosclerosis plaques back to the liver where it is excreted by metabolism (von Eckardstein 2001). On top of this athero-protective function, the HDL has many functions as anti-inflammatory, immunosuppressive functions, antioxidant and vasodilator capabilities (Tall 2008). Furthermore it is discovered that these antiatherogenic effects of HDL depends to a large extent on its biological quality, namely all HDL subclasses mentioned above have different and important roles in athero-protective function (von Eckardstein 2001).
2. Homeostasis of Cholesterol and its Metabolic Pathways

Cholesterol homeostasis is regulated by a balance of de novo synthesis, catabolism in the liver, intestinal absorption, secretion into bile and fecal excretion. Physiologically, the amount of cholesterol secreted into bile each day is similar to the amounts synthesized in the liver and absorbed from the intestine (Vuoristo 1994).
In order to satisfy the daily requirement, adult men produce 1 gram of cholesterol through mevalonate route, an enzymatic pathway starting with the enzyme acetyl-CoA to obtain the final steroid. This endogenous cholesterol is produced in situ in each tissue or in the hepatocytes. Also an important source of cholesterol is the diet (Incardona 2000).

2.1 Homeostasis of Cholesterol into the Gut and its Regulation

Lipids coming from the diet are a mixture of triglycerides, phospholipid and cholesterol, which are barely soluble in aqueous environments. Therefore lipids, partially digest by gastric lipases, create in the gut an emulsion called micelles. Micelles are lipid molecules that arrange themselves in a spherical form in aqueous solutions. Fatty acids are important elements of micelles, which are amphipathic molecules mainly composed by hydrophilic (polar head groups) and hydrophobic regions (long hydrophobic chain). Micelles contain polar head groups that usually form the outside of the micelle surface. The hydrophobic tails are inside and away from the water since they are nonpolar (Seddon 1995). Micelles are involved in the dietary cholesterol absorption, interacting with various receptors situated on surface of villi take place into the duodenum and the jejunum. Layer of mucus, enveloping the gut lumen, is main restriction of cholesterol absorption into the enterocytes. Mostly composed with glycoproteins as mucina, it has one unit of pH more acid compared with rest of gut. This facilitates fatty acids uptake since protonated molecules are more soluble than ionised ones (Mansbach II 2012).
The net intestinal cholesterol absorption is the difference between two dissimilar mechanisms: cholesterol uptake from enterocytes and its excretion into the intestinal
lumen. Former mechanism is regulated mostly by receptor Niemann–Pick C1 Like 1 (NPC1L1) for not esterified cholesterol, whereas for esterified cholesterol by aqueous diffusion. Latter mechanism is controlled by the cholesterol transporters ATP-Binding Cassettes type G5/8 (Figure 4) (Turley 2008).

- **Intestinal absorption of diet-derived cholesterol and its pathway of hepatic metabolism**

Intestine receives lipids and cholesterol mainly from food, bile secretion and, in small amount, from the desquamation suffered by its epithelium (Davis 2011). Interaction between free cholesterol and NPC1L1 is highly selective, unlike other sterols; this protein is expressed on cellular brush border of the proximal jejunum. The human NPC1L1 contains 1332 amino acids and 13 trans-membrane domains, with three loop side facing the intestinal lumen, which have high affinity with sterols (Davis 2009).

Altmann et al identified NPC1L1 as a critical protein for absorption of dietary and biliary cholesterol. These studies showed that deletion of NPC1L1 in mice resulted in a reduction of almost 69% of cholesterol absorption (Altmann 2004). Later, it was further discovered that NPC1L1 can transport phytosterols, as well as cholesterol, thus it has been affirmed that it is a key modulator of whole body cholesterol homeostasis (Davis 2004).

As mentioned above, cholesterol reaches the intestinal villi on the membrane of enterocyte through micelles, which interact with NPC1L1 to release cholesterol; in order to facilitate the absorption of esterified cholesterol, beforehand it is hydrolysed.
by the cholesterol ester hydrolase enzymes (Grenier 2012), and only a small part of sterol may pass the endothelium without changes. As shown in figure 4, cholesterol proceeds through the membrane of enterocytes, then it heads to the endoplasmic reticulum to be esterified by the acetyl-coenzyme A acetyltransferases (ACAT); subsequently, it is incorporated along other lipids and lipoproteins Apo A-I, A-II, C-I, C-II, CIII, E and B-48, into nascent chylomicrons particles (CM) (Turley 2008).

- **Cholesterol efflux from enterocytes**

In order to protect the organism by cholesterol excess, the heterodimer of intestinal transporters ABCG5/8 leads to cholesterol excretion in the intestinal lumen, with opposite direction of cholesterol flow NPC1L1 mediated (figure 4). Indeed it has been showed that this heterodimer mediates the efflux of cholesterol and other sterols from blood circulation to intestinal lumen, via enterocytes (Brown 2009). Both ABCG5 (sterolin-1) and ABCG8 (sterolin-2) are composed by an ATP-binding cassette transporters with N-terminus, a trans-membrane domain containing six helices, and a nucleotide binding domain (NBD) (Brown 2010). A variety of studies showed that both transporters heterodimerize in the endoplasmic reticulum (ER), and move together, through the Golgi apparatus, to apical layer of plasma membrane (Calandra 2011). To obtain the sterol efflux capacity ABCG5 and ABCG8 must work together, creating a heterodimer. Plosch et al and Wang et al have demonstrated that the lack of either ABCG5 or ABCG8 in mice leads to low cholesterol concentrations in bile, whereas biliary phospholipids and bile acid concentrations are not altered, so this reveal the transport selectivity of the heterodimer (Plosch 2004; Wang 2007). Conversely, overexpression of ABCG5 and ABCG8 in mice increases biliary cholesterol levels by more than fivefold (Yu 2014). The dimer formed by two transcription factors, Liver X Receptor (LXR) and retinoid X Receptor (RXR), modulates the expression of genes of ABCG5/8 and therefore the transcription of the transporters mentioned above.

### 2.1.1 Bile Synthesis and Enterohepatic Circulation

Bile salts are produced by hepatocytes, which use cholesterol for bile synthesis (approximately 500 mg). Bile is important for the digestion process, because its main
Introduction – *Homeostasis of Cholesterol and its Metabolic Pathways*

constituent, bile acids, permits the creation of micelles, involved in absorption of lipids and lipophilic vitamins as A-D-E-K from intestine. In addition to these functions, bile promotes the elimination of bilirubin, metabolite of haemoglobin, and it neutralizes surplus of acidity in the small intestine, this due to the digested nutrients from stomach (Russell 2003).

Bile also called bile salts consist mainly of sulphate conjugates of bile alcohols and of taurine (or glycine) conjugates with bile acids (Dawson 2006). These acids are synthesized from cholesterol in the pericentral hepatocytes of the hepatic acini. In this process, the hydrophobic cholesterol is converted in water soluble, amphipathic product through a series of sterol ring hydroxylations and oxidation steps. Bile acids formed in the hepatocytes are called primary bile acids to distinguish them from secondary bile acids, which are formed through enzymatic activity of intestinal bacteria, modifying the oxyl groups of main components of bile acids in humans, the cholic acid (CA) and the chenodeoxycholic acid (CDCA), by dehydrogenation or dehydroxylation (Zwicker 2013).

The complex process of primary bile acids biosynthesis requires the action of 17 different enzymes. Two biosynthetic pathways, termed “classical” and “alternative” pathways, are main responsible for bile acid formation, although several other minor routes have been described, which in some species and situations may also have relevance (Axelson 2000). The classical pathway (CYP7A1 pathway) occurs solely in the liver, it is also known as the “neutral” pathway because its intermediate metabolites are neutral sterols (CA and CDCA). Then the “alternative” pathway (CYP7B1 pathway), called acidic pathway, because its intermediate metabolites are acidic (Russell 2003).

Final step of neutral pathway, leads to the conjugation of the terminal side-chain carboxylic acid with the amino acids glycine or taurine mediated by enzyme CYP7A1, which converts cholesterol directly into 7α-hydroxycholesterol. Conversely, in the alternative acidic pathway, cholesterol is first converted by C-24, C-25, or C-27 sterol hydroxylases into oxysterols by CYP27A1. Afterward the oxysterol 7α-hydroxylase (CYP7B1) converts the mentioned sterols in 7α,27-dihydroxycholesterol (Dawson 2006). At the end of both pathways the newly synthetized bile acids are conjugated with an amino acid and carried in gallbladder.
However, the exact order of steps in the biosynthetic pathway remains unclear, since many intermediates serve as substrates for more than one biosynthetic enzyme (Russell 2003). The activities of both pathways are strictly regulated. Indeed when bile is piling up in the gallbladder, the synthesis is reduced by a negative feedback mechanism that decreases the expression of CYP7A1 and CYP8B1. Conversely, cholesterol accumulation induces bile acid synthesis by activating CYP7A1. The regulatory responses of CYP7A1 are mediated at the transcriptional level: suppression is triggered by bile acids binding to the farnesoid X receptor (FXR), whereas enhancement of transcription is mediated by the liver X receptor (LXR) (Bonamassa 2013).

Bile amount produced by organism is insufficient for daily requirement. Therefore the mammalians need a physiological mechanism to recover bile salts termed enterohepatic circulation. As showed in figure 5, newly synthesized bile acids are secreted into the bile, afterwards delivered into the lumen of the small intestine. Later, bile acids can be transported from the intestine to the liver via the portal circulation and re-secreted into the bile (Cai 2014). Through such enterohepatic circulation, about 95% of bile acids are recovered by the gut and only 5% are lost and replaced by new synthesis in the liver.

The enterohepatic circulation is divided into two independent pathways, the portal and extraportal pathways. The extraportal enterohepatic circulation consists primarily of lymphatic drainage of bile from the intestine to liver, via chylomicrons, than it is carried into the superior vena cava. This extraportal pathway plays little role in the enterohepatic circulation of bile acids. Indeed 95% of bile reabsorption is mediated by portal pathway, whereby bile is absorbed by enterocytes of ileum and delivered in portal vena (Dawson 2006). On apical membrane of enterocytes, the apical sodium-dependent bile salt transporter (ASBT) mediates the reabsorption of bile salts from intestine. Then they interact with a cytoplasmic protein, ileal lipid binding protein (ILBD), called gastrotropina, which carries bile salts to basolateral membrane, where two different transporters lead them into the portal vena. The mechanism is not entirely clarified. As supposed, that bile salts reach portal circulation through the transporters Multidrug resistance-associated protein 3 (or ABCB4) or by Organic Solute Transporters (OST) α and β. Once in portal circulation, the bile salts are
delivered into hepatocytes through sodium-dependent hepatocyte bile salt uptake system (NTCP), a transporter expressed on the sinusoidal domain of hepatocytes (figure 5) (Balakrishnan 2006).

In the liver, bile salts are carried in bile duct where, losing water and electrolytes become 10% more concentrated; afterwards they are stored in gallbladder. Responsible for bile salts transport is the heterodimer ABCG5/8 presents on the cellular membrane surface of bile duct. These transporters are also involved in the cholesterol efflux from enterocytes mentioned above (Escolà-Gil 2014).

As in the enterocytes, the activity in hepatocytes of ABCG5/8 is traded off by activity of NPC1L1, which, supposedly, has function to capture cholesterol from bile and carries it in hepatocytes, so as to avoid excessive leak of the sterol (Bonamassa 2013).

Figure 5: Transport of bile acids within the enterohepatic circulation and excretion out of the body. Bile acids are synthesized by the liver and released into the lumen of the small intestine via bile (Zwicker 2013)
2.2 Metabolic Pathways of Plasmatic Cholesterol

Cholesterol and lipids circulating in blood are metabolized by two different pathways: endogenous and exogenous lipid transport, both mediated by lipoproteins.

2.2.1 Exogenous Pathway

Exogenous pathway, also called extraportal pathway mentioned above, is responsible of cholesterol metabolism from diet-derived lipids. As described before the chylomicrons newly synthetized from enterocytes, lead cholesterol and lipids from the intestine to liver, through lymphatic system and the thoracic duct to drain finally in the systemic circulation (Castro-Torres 2014). Here, the chylomicrons, rich in triglycerides, are hydrolysed from Lipoprotein Lipase (LPL), and the released fatty acids are used by peripheral tissues as energy source. Afterward, ApoE on the surface of remnants chylomicrons allows hepatic uptake mediated by LDL receptors, LDL receptor related protein-1, heparin sulphate proteoglycans and Scavenger Receptors Type I (Mahley 1999).

2.2.2 Endogenous Pathway

Purpose of endogenous pathway is the metabolism of VLDL newly synthetized by liver or derived from HDL. VLDLs, are assembled by the enzyme microsomal transfer protein (MTP) and contain ApoB-100, ApoE, ApoC e triglycerides. In the blood the triglycerides are hydrolysed by LPL and phospholipids are transferred to HDL by phospholipid transfer protein (PLTP) (Vance 2002). Consequently, these factors induce the formation of IDL, which may be or uptaken by the liver or remodelled by hepatic lipase (HP) to generate LDL. Then cholesterol-enriched LDL may bind with LDL receptor in the liver and in peripheral tissues. Homeostatic levels of cholesterol in tissue are under control of a feedback regulatory pathway. If the accumulation of lipids in peripheral tissue increases, the enzyme sterol regulatory element binding proteins (SREBP) modulates the transcription of genes involved in sterol and fatty acids biosynthesis and uptake (Mansbach II 2012).
2.3 Excretion Pathways of Cholesterol and Plasmatic Lipids

Toxic excess of cholesterol cannot be catabolized to a significant extent in mammals, therefore excretion is the predominant way to disposal it. As mentioned in literature, plasma cholesterol is eliminated via several pathways: minimal part is eliminated via skin and intestinal cell loss; conversely majority of cholesterol is excreted via other two ways. First pathway is reverse cholesterol transport (RCT), where cholesterol is removed from peripherals tissues, delivered by HDL into blood and finally, metabolized by liver into the faces as neutral sterols or bile acids (Favari 2015). Recently, many studies have provided evidence for another metabolic pathway, which does not involve hepatobiliary route, but rather cholesterol excretion occurs directly from plasma to intestine lumen through enterocytes. This mechanism is termed trans intestinal cholesterol excretion (TICE) (Tietge 2013).

2.3.1 Reverse Cholesterol Transport (RCT)

RCT was first described by Glomset as the physiological pathway whereby cholesterol is removed from peripheral tissues, including foam cells, delivered by HDL into blood and finally uptaken by liver for disposal into faeces (Glomset 1968). RCT may counteract the formation and development of atheroma, by promoting HDL-mediated removal of cholesterol from the artery wall. As recent studies in vivo established, RCT is inversely correlated with cardiovascular disease (CVD). Thus suggests the promotion of this physiological mechanism may be a strategy to decrease atherosclerosis plaque, therefore decreasing CVD (Rader 2009). Indeed, several pharmacological modulations on the RCT process has been extensively investigated (Zanotti 2008).

This mechanism could be summarized in three steps (Figure 6):

1. cellular cholesterol efflux from macrophages of atheroma;
2. HDL remodelling in plasma;
3. Cholesterol hepatic uptake and faeces excretion.

- First step:

Macrophages can protect themselves against cholesterol toxicity by converting free cholesterol to cholesteryl esters or by effluxing cholesterol to extracellular acceptors.
As mentioned above, macrophages are relevant cells in atheroma, responsible of cholesterol efflux from plaque to plasma lipoproteins, namely the first step of RCT (Zanotti 2012).

Cholesterol efflux is dependent on cholesterol amount into the macrophages, on the expression of cholesterol transporters and on different lipid composition and dimension of HDL, whereby the levels of these particles play key role to determine the efficiency of cholesterol efflux. Indeed, in literature, a variety of specific pathways of cholesterol efflux, depending on interaction among transporters and lipoproteins, have been defined:

a. efflux to lipid-poor apolipoproteins, particularly ApoA-I, mediated by ATP-Binding Cassettes A1 (ABCA1);

b. efflux to mature HDL particles mediated by ATP-Binding Cassettes G1(ABCG1);

c. efflux to mature HDL particles by scavenger receptor class B type I (SR-BI) as well as passive diffusion.

In particular, ABCA1 is unidirectional ATP-depending transporter that carries out cholesterol against concentration gradient. It interacts with lipid free ApoA1 generating discoidal HDL particles (preβ-HDL). Conversely ABCG1 and SR-BI, interacts with mature HDL. Former is another ATP-depending transporter, while SR-BI mediates a bidirectional flux of free cholesterol, whose net movement is determined by the concentration gradient (Tall 2008).

In addition of transporter-mediated efflux, passive diffusion is the simplest bidirectional efflux mechanism, since it does not require cellular energy. Cholesterol may pass through the membrane because it is sufficiently water soluble to be transferred to extracellular acceptors (Rothblat 2010).

The contribution of transporters to cholesterol efflux from macrophages has been clearly quantified in vitro. Adorni et al. demonstrated that in cholesterol loaded macrophages of mice (murine peritoneal macrophages, MPM) ABCA1 contribution is predominant and represents 35% of total efflux, ABCG1 contributes less than ABCA1 (21%); while SR-BI contribution is minimal (Adorni 2007). As Larrede et al demonstrated, these relations are maintained in humans, in particular ABCA1 still remains the predominant contributor to cholesterol efflux in cholesterol loaded
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human macrophages but, differently from mice, SR-BI is relatively more important than ABCG1 (Larrede 2009).

Fielding at al. first has affirmed that from sera of different humans have different ability to promote cholesterol efflux (Fielding 1983). Later it has been demonstrated in several studies that serum HDL (serum depleted of apoB-containing lipoproteins) from different humans have different efflux capacities, despite their similar levels of HDL-C (de la Llera-Moya 2010). The potential clinical relevance of this observation was demonstrated by analysing samples from two large-cohorts. Impaired efflux capacity of HDL was a superior predictor of CVD status than traditional CVD risk factors. Indeed, efflux capacity strongly and negatively associated with CVD status after correction for HDL-C levels (Khera 2011). Moreover, ApoAI explained only 40% of the variance in efflux capacity, while HDL-C accounted for only 34%. These observations strongly support the proposal that HDL’s ability to remove cholesterol from macrophages is important for human cardio protection (Hutchins 2015).

It is not completely clear how HDL enter into the atherosclerotic plaque and reach the intima to interact with macrophages, and also the way of HDL to return into the circulation system.

von Eckardstein A. et al described the activity of endothelial cells to internalize and transport HDL and lipid free ApoAI into the plaque and vice versa, after cholesterol loading, into the blood. This saturable mechanism consists in HDL and lipid free ApoAI interactions with ABCA1, SR-BI and endothelial lipase and their carriage through endothelial cell layer via transcytosis mediated by ABCG1 (Rohrer 2009). Furthermore, the same researcher team identified another mechanism, whereby ApoAI binds with β-chain of F₀F₁-ATPase expressed on endothelial cells. This interaction stimulates ATP hydrolysis, and the generated ADP selectively activates the purinergic receptor P2Y₁₂, leading to internalization and transport of lipid-free apoA1 as well as HDL (Cavelier 2012). Once apoA-I and HDL have been loaded with cholesterol, they may return from the extravascular space to the systemic circulation in two different ways: passing through the endothelium or via the lymphatic system (Rohrer 2006).
- **Second step:**

In the blood, cholesterol enriched HDL may modify their size and composition, thus the capacity of promoting RCT, through the activity of two different enzymes: lecithin-cholesterol acyltransferase (LCAT) are cholesteryl ester transfer protein (CETP).

LCAT is a plasma glycoprotein synthesized by the liver, which esterifies free cholesterol. In particular it enriches nascent discoid HDL with cholesteryl esters, transforming it in a large spherical mature HDL. The enzyme activity maintains difference of concentrations among the hydrophobic core and the lipoprotein surface, in order to ensure the interaction between cells and HDL, which is necessary to drive the trafficking of cholesterol (Calabresi 2011). Despite the central role of LCAT in HDL remodelling, its contribution in development of atheroma is not clearly defined yet. As shown in literature, overexpression of the enzyme in mice leads to unaffected or even accelerated atherosclerosis. However, Furbee and co-workers, has conducted tests with LDLr-/- mice, compared with ApoE-/- mice, both models without LCAT enzyme. These experiments showed that LCAT deficiency in LDLr-/- and ApoE-/- mice fed an atherogenic diet resulted in increased aortic cholesterol deposition, likely due to a reduction in plasma HDL, an increased saturation of cholesteryl esters in apoB lipoproteins and, in the ApoE-/- background, an increased plasma concentration of apoB lipoproteins (Furbee 2002).

As shown in figure 6, LCAT functions exert when free cholesterol and phospholipids are mobilized from macrophages to Apo AI particles which turn into discoid pre-β HDL. The esterified cholesterol enrichment, of pre-β HDLs, mediated by LCAT results in forming large spherical mature HDL3 and HDL2 (Dieplinger 1985). Afterwards, ABCG1 and SR-BI, present on macrophage surface, interact with HDL3 and HDL2, contributing to cholesterol efflux (Santos-Gallego 2011). Within HDL, cholesterol is directly delivered to liver where is uptaken through SR-BI, whereas small cholesterol depleted HDL particles are released and could be recycled into plasma for lipoprotein assembly (Calabresi 2011). After hepatic metabolism, cholesterol is excreted as neutral sterol by ABCG5/8 (Escolà-Gil 2014).

Second enzyme involved in first step of RCT is the CETP. This enzyme is a hydrophobic glycoprotein secreted mainly by the liver. It binds principally HDL and
also it promotes the redistribution of cholesteryl esters, triglycerides and phospholipids between lipoproteins. In particular it promotes a net mass transfer of cholesteryl esters from HDL to Apo B containing lipoproteins (mainly LDL) and of triglycerides in the opposite direction. This protein is present in humans, rabbits and hamsters but is not expressed in mice and rats. It has been observed in Japanese population, that absence of this enzyme, due to homozygous mutation, causes elevations in HDL-C and apoA1 levels and a moderate reduction in LDL-C and apoB levels. On the basis of this phenotype, CETP inhibition was proposed as a possible strategy to increase HDL levels in humans and to reduce atherosclerosis. However, researches regarding the consequences of CETP activity in the RCT pathway has provided ambiguous data, as both elevation as well as inhibition of CETP activity can be favourable (Barter 2003). ApoB-rich lipoproteins subsequently bind to hepatic LDL receptor thus completing the RCT cycle (Santos-Gallego 2011).

- ***Third step***

Free and esterified cholesterol of HDL2 and 3 are selectively uptaken by SR-BI transporters in the liver, resulting in cholesterol-poor HDL that can be recycled (Rader 2009). SR-BI interacts mostly with ApoAI particles forming the HDL 3 and 2. In addition, HDL2 can be recognized by LDL receptor situated on hepatocytes surface (van der Velde 2010b). After transferring of cholesteryl esters from HDL to lipoproteins containing ApoB particles via CEPT, LDL can be internalized in hepatocytes, via LDL receptors. After hepatic uptake, cholesterol may be excreted in the bile via ABCG5/ABCG8 as neutral sterol or via ABCA11, after conversion into bile acids, so as to be eliminated via faeces. Bile salts are secreted from the gallbladder in the duodenum region of the intestine (Yu 2002).
2.3.2 Trans Intestinal Cholesterol Efflux (TICE)

For a long time the hepato-biliary secretion (last step of RCT) was considered the only route to cholesterol metabolism. Conversely, in the last few years a non-biliary cholesterol secretion pathway has discovered, in which the intestine plays a main role. This pathway is termed Transintestinal Cholesterol Efflux (TICE), which contributes significantly to the total faecal neutral sterol excretion (van der Velde 2010a).

TICE has been proposed in 1927, by Sperry et al. but has never been proven directly until recently. Indeed Pertsemlidis et al. confirmed the data of Sperry almost half a century later, also in studies with dogs (Pertsemlidis 1973). The role of intestinal transporters ABCG5/ABCG8 and ABCB4, involved in enterohepatic circulation, is the secretion of cholesterol into the bile; therefore inhibition of these transporters should result in a drastic lowering of faecal neutral sterol excretion. However, experiments performed with Abcg5/Abcg8 double knockout mice and Abcb4 knockout mice do not show low levels of faecal neutral sterols. These data suggest
that in addition to biliary secretion, another excretion route of cholesterol exist (van der Velde 2007). In human, faecal sterols of non-dietary origin are present in faces of patients with biliary obstruction (Cheng 1959).

TICE consists in cholesterol excretion directly from peripheral tissues to intestinal lumen, going through enterocytes. It takes place mostly in the proximal 10 cm of small intestine and, as showed by different studies, the high-fat diet, plant sterols, and liver X receptor activation (PPARδ ligands) may stimulate it (Tietge and Groen 2013).

The mechanism of TICE is not well understood. It is supposed that the cholesterol pool of the intestinal cells is due to uptake by NPC1L1 from the intestinal lumen, by the endogenous synthesis, and by uptake from HDL and LDL on the basolateral side (blood compartment). Le May et al demonstrated that the main cholesterol donor for TICE in the blood compartment are LDL, which interact with LDL receptor and SRBI, both expressed in basolateral face of enterocytes (Le May 2013). After cholesterol uptake in enterocytes, many intracellular transporters are involved in cholesterol trafficking from basolateral to apical side, as the intracellular transporter Rab9, which plays a role in cholesterol trafficking from late endosomes to the trans-Golgi network. In addition, the lysosomal integral membrane protein 2 (Limp2) in mice and the orthologous human scavenger receptor class B type 2 (Scarb2) influence cholesterol homeostasis, acting upon intracellular vesicular trafficking (van der Velde 2010a). What the exact relationship between these two factors and TICE might require further studies.

Cholesterol delivery from enterocytes to intestinal lumen may be mediated by the heterodimer ABCG5/G8. Although it may be a likely candidate for last step of TICE, initial studies in mice lacking functional ABCG5/G8, as mentioned above, have showed that TICE was not reduced in Abcg8-/- mice, hence a possible function for ABCG5/G8 in TICE was questionable. However, a more recent study from van der Veen et al demonstrated that increase in TICE upon LXR agonist treatment was ABCG5/G8 dependent (van der Veen 2009).
3. Gut Microbiota and its Role in Atherosclerosis

As described above, intestine plays an important role in the metabolism of cholesterol and lipids. Indeed it is involved in all physiological mechanisms aimed to regulate cholesterol homeostasis. Gut functions on cholesterol regulation and dietary nutrient absorptions are partially due to the heterogeneous population of bacteria termed intestinal microbiota, which lives in suspension into the mucus enveloping the intestinal lumen. The relationship between the intestinal microbiota and the host is usually denoted as commensal (one partner benefits without negatively affecting the other partner) (Marik 2012). Furthermore, microbiota also provides a multitude of others functions for the host, as production of group B and K vitamins and short-chain fatty acids (SCFA), involved in cholesterol homeostasis, the fermentation of indigestible food substances and the prevention of luminal colonization by pathogenic bacteria (Escherichia coli, Clostridia, Salmonella, and Shigella species) (Zoetendal 2001).

3.1 Overview of Gut Microbiota: Composition and Origin

Intestinal lumen is the principal location of microbiota, a complex ecosystem mostly composed of bacteria, that colonizes mucosal layer of large intestine, reaching the density of \(10^{11}\) cells/ml of luminal content (Turroni 2008). Gut is one of the richest environments inside the human organism, colonized by almost 100 trillions of bacteria, in which 99% of resident bacteria are anaerobic organism, resulting by a selective process that has led to the creation of a stable microbial ecosystem with a high degree of functionality (Gritz 2015). Furthermore it has been estimated that the cumulative genome of all these microorganisms contains an amount of genes 100 times greater than whole human genome (Org 2015). In addition, bacteria residing in the gut, account for 60% of faecal mass (Adlerberth 2009).

Despite its importance, the precise composition and activities of the intestinal flora in human are still subject of intensive studies, using culture-based methodologies and, above all, the metagenomic approach. This technology consists in the amplification and sequencing of small subunit ribosomal DNA sequences of complex and
heterogeneous bacterial communities, combined with functional analyses of all microbial genomes contained within intestinal lumen (Gill 2006).

To get an overview of microbiota composition, it has been conducted two different studies, the Human Microbiome Project (HMP) in the U.S.A. and the European Metagenomics of Human Intestine (metaHIT), consisting in a metagenomic analyses of mucosal as well as fecal samples. These studies revealed the presence of representatives of the phyla Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, and Actinobacteria (Eckburg 2006). With a relative abundance of 25 and 65%, respectively, Bacteroidetes and Firmicutes represent together up to 90% of the total microbiota, whereas Actinobacteria, Proteobacteria, and Fusobacteria are the subdominant phyla with a relative abundance respectively of about 5, 8, and 1%, (Candela 2010). As showed in literature, qualitative and quantitative microbiota profile changes person to person, so much so it has been introduced the concept of “microbiota fingerprint” to identify the univocal composition of gut microbiota in each person. Arumugam et al demonstrated in a meta-analysis study, that people of different countries have a common core of bacterial strains of three genera: Bacteroides, Ruminococcus and Prevotella (Arumugam 2011). Furthermore, bacteria of core are considered resident microorganisms (mucosa-adherent components), whereas others are transient bacteria, which come from the diet and other environmental factors (Turroni et al. 2008).

The “microbiota fingerprint” depends on several factors:

- Genetics factors;
- vertical transmission of maternal microbiota;
- delivery types;
- type of diet and breast feeding;
- gastrointestinal disorders and antibiotics administration.

a. The effect of genotype on the composition of the human microbiota was demonstrated in a study involving monozygotic twins (Zoetendal 2001). Zoetendal et al. showed that there were more similar strains between microbiota of monozygotic twins than between twins and their unrelated marital partners.
b. The most important contribution to the genesis of the microbiota is vertical transmission of maternal microbiota. Massive colonization of mucosa in the digestive, respiratory, urogenital tracts and the skin occurs during delivery, when the infant is in contact with mother’s microbiota. It has been previously proposed that the uterus and the placenta were largely sterile and that a foetus was not colonized with bacteria until the time of birth. Conversely, Aagaard et al. demonstrated the presence of a microbiome within the placenta, suggesting that the colonization process begins well before delivery (Aagaard 2014). In particular normal colonization of the sterile intestine is a complex process, starting with the establishment of facultative anaerobes bacteria (Escherichia coli and Streptococcus) within few hours after birth, reaching concentrations of $10^8$ to $10^{10}$ /g of faces after 3,3 days (Adlerberth and Wold 2009). By about day 10, all newborn display colonization by a heterogeneous bacterial microbiota, in particular by bifidobacteria species, this due consumption of breast milk, rich in these bacteria. During the first month, bifidobacteria and E coli are the predominant bacteria, as well as Lactobacillus, Bacteroides, all in similar quantities. After about 1 year, Lactobacillus, Bacteroides, and clostridia species increase, while bifidobacteria and E. coli decrease; the microbiota composition of 1 year old infant resembles that of the adult, and the main bacterial groups isolated are Staphylococcus, Streptococcus and Enterobacteriaceae species (Thompson-Chagoyán 2007).

c. The initial microbiota profile of newborn much depends on type of delivery. In particular infants born via vaginal delivery, after 4-6 days, have intestinal colonization reflecting maternal vaginal flora, as the anaerobic Lactobacillus and Prevotella species. Conversely, infants born via Cesarean delivery are colonized by epidermal instead of vaginal species, such as Clostridium, Staphylococcus, Propionobacterium, and Corynebacterium, and only 9% of those born have the anaerobic bacteria of vaginal delivery infants (McFarland 2000). Moreover, they have a deficiency of the anaerobes species Bacteroides and Bifidobacterium compared with the previous infants (Gritz 2015).
Jakobsson et al. demonstrated that infants delivered via Cesarean section displayed delayed gut colonization in particular by members of the Bacteroidetes phylum until 1 year (Jakobsson 2014). Other studies have also shown persistent differences in intestinal microbial colonization between Cesarean-delivered and vaginally delivered children as far as 7 years of age (Salminen 2004).

d. Diet is the second important factor contributing to the modulation and genesis of microbiota. In particular it has been observed that, in the first stages of life, the type of diet (breast vs. formula feeding) markedly affects the colonization pattern (Harmsen 2000). Human milk contains almost $10^9$ bacteria/litre; therefore in breast-fed babies, milk promotes intestinal growth of bifidobacteria and lactobacilli. Moreover the frequent contacts with nipple facilitate the growth of skin-specific strains as Staphylococcus, Streptococcus, Corynebacterium, Lactobacillus, Micrococcus, Propionibacterium, and Bifidobacterium (Gronlund 2000). Conversely, the profile of formula-fed infants is much more similar to that of adults. Indeed, into the faeces of these newborn, higher levels of anaerobic strains typical of adult microbiota are found, as Staphylococcus, Streptococcus, Enterobacteriaceae, and Bifidobacterium colonization occurs several days later (McFarland 2000). During the weaning, the microbiota changes its composition, resembling that of adults, because of the introduction of solid food in infant’s diet. Within the intestine of breast-fed infants a major disturbance in the microbial ecology occurs, with a sharp increase of Enterobacteriaceae and colonization by Bacteroides and Clostridia species (Stark 1982). Furthermore, it has also been reported that staphylococcal colonization rates remain higher in breast-fed, this probably due to prolonged contact with maternal skin during suckling. These events are not observed when formula-fed infants ingest solids foods (Thompson-Chagoyán 2007).
3.2 Functions of Gut Microbiota within the Host

The functions of the normal flora have been called microflora-associated characteristics’ (MAC) by majority of researchers. These MACs include the digestion of substrate as fibres, oligosaccharides, sugars, some lipids and proteins to obtain nutrients for bacterial cells; as well as, within the colon, another source of nutrients for bacteria includes mucin, epithelial and enterocyte tissues, bile acids and cholesterol. The changes of balance among food nutrients encourage the adaptation of strains able to survive with the nutrients presents in the intestine rather than others which are not able (McFarland 2000).

Main products of bacterial digestion of carbohydrates are short chain fatty acids (SCFAs) as acetic, propionic and butyric acids. All of these are used by bacteria as additional energy source. Furthermore propionic acid is involved in the control of hepatic cholesterol synthesis, whereas butyric acid has several functions including the maintenance of the integrity of the colonic epithelial layer and source of energy for human colon epithelial cells (Ooi 2010; Clausen 1991). Treem et al. studied the SCFA producing activity of intestinal flora in patients with Inflammatory Bowel Disease (IBD) compared with control patients. It has been showed that patients with IBD produced less total SCFA, less acetate acid and less propionic acid. Since normal flora is responsible for the production of SCFA, this study may indirectly link normal flora disruption with IBD (Treem 1996).

Intestinal flora is deemed to be the first defence line against the pathogenic organisms and indigenous opportunist organisms living within the gut. Colonization resistance is a dynamic phenomenon, whereby intestinal flora impedes pathogenic colonization since native bacteria have major adaptation to intestinal environment. Indeed an important mechanism for colonization resistance are competition for nutrients, as well as the production of metabolic products, as the $\text{H}_2\text{O}_2$ results in peroxidation of lipid membranes, increased bacterial membrane permeability, destruction of bacterial nuclear acids in bacterial strains that do not possess catalase (Aurora 2015). Colonization resistance has been found to be an extremely effective natural barrier against pathogens as C. difficile, Salmonella, Shigella, Pseudomonas, E. coli strains, Candida albicans and others (Apperloo-Renkema 1990).
Gut microbiota provides the daily requirement of several vitamins including Vitamin K, panthothenic acid (B5), Vitamins B12, pyridoxine (vitamin B6), biotin (vitamin H), niacin, riboflavin and thiamin. In absence of microbiota, vitamins mentioned above would not be produced or transformed in absorbable form (Patterson 2014). Normal flora also induces the maturation of the gut-associated lymphoid system (GALT), which control the migration of B-lymphocytes from peritoneum to intestinal surface and their maturation in plasma cells, in turn producing Immunoglobulin A (IgA) (Tsuji 2008). Moreover Pulverer et al. found that intestinal bacteria releases low molecular weight substances that interact with MALT (mucosa associated lymphoid tissue) and these substances appear to be essential for an adequate immune response. As showed in Pulverer’s experiments, the absence of microbiota in a mouse model, due to an antibiotic treatment, resulted in a decreased immune response (Pulverer 1997).

3.3 Relationships of Microbiota and its Metabolites with Atherosclerosis and Inflammation Process

Recent data demonstrate that intestinal flora influences lipid metabolism and other factors involved in the development of metabolic and cardiovascular diseases. As showed in figure 7, microbiota is composed of different strains associated with several effects on the host; therefore part of intestinal flora may have atheroprotective effects, while other part may have proatherogenic effects (Goldsmith 2014).

- Antiatherogenic effects

As shown in figure 7, accumulated data revealed a close relationship of microbiota with the positive modulation of lipid metabolism and inflammatory pathways, both atherogenic factors. Stepankova et al. demonstrated the injection of normal human microbiota in germ free ApoE knockout mice, prevents atherogenesis, even if this animals were fed with a standard low-cholesterol diet (Stepankova 2010).

An interesting function of gut microbiota is the fermentation of food-derived indigestible carbohydrates, that results in a production of several metabolites named short-chain fatty acids (SCFA). These molecules are used by bacteria as primary
energy source (Wong 2006), and play an important role in the cholesterol reduction. The SCFA production in the large intestine is 100 to 450 mmol per day, with relative proportions of acetate, propionate, and butyrate of 60:20:15. These SCFAs have different effects in the host. Indeed acetate in the serum seems to increase total cholesterol, while propionate increases blood glucose and tends to lower the hypocholesterolemic response caused by acetate, which reduces its utilization by the liver for fatty acid and cholesterol synthesis (St-Onge 2000).

SCFAs have been reported to act as ligands that bind to activate peroxisome proliferator-activated receptors (PPARs). PPARγ controls the transcription and translation of angiopoietin-like protein 4 (ANGPTL4), which is a LPL inhibitor. As mentioned in previous chapters, LPL promotes the storage of VLDL and chylomicron triglycerides in adipose tissues. Mainly activators of PPARγ are butyrates, propionate, and acetate, which induce a suppression of LPL activities via activation of ANGPTL4. This regulates fatty acid oxidation in muscle and adipocytes that results in a reduction in fat storage (Korecka 2013).

Recent findings showed that the metabolism of diet-derived anthocyanin by microbiota could be an atheroprotective effect, as the protocatechuic acid (PCA), that is directly produced by gut microbiota from the metabolism of cyanidin-3-O-β-glucoside (α-G), compound mostly found in blackberries and bilberries (Wang 2010). PCA was found to promote cholesterol efflux from macrophages through activation of expression of ABCA1 and ABCG1 by down-regulating microRNA (miR)-10b that interrupts both cholesterol transporters synthesis (Kontush 2015). In addition, Cy-3-G was able to improve serum cholesterol levels in ApoE-null mice and increase formation of bile acids through activation of liver expression of CYP7A1 via direct binding to LXRα (Wang 2012).

- **Proatherogenic effects**
  
  As showed in literature, intestinal flora is considered an agent activating inflammatory mechanisms. Indeed microbiota may stimulate infiltration of macrophages in the adipose tissue mediated by several stimuli as the lipopolysaccharide (LPS) endotoxin and SCFA, both produced by bacteria (Bäckhed 2004). LPS lead to a chronic inflammatory state and contribute to progression of
metabolic diseases, such as obesity, hyperlipidaemia, and other risk factors of atherosclerosis (Jialal 2014).

LPS could be delivered from the gut to the circulation through chylomicron-associated transport and via tight junctions of enterocytes (Caesar 2010). In animal models simulating obesity condition in humans, the association of LPS with the rearrangement of tight junction proteins, the reduction of epithelial barrier function, the increased gut permeability, endotoxemia, and inflammation have been observed (Brun 2007). The inflammatory state induces an increase of intestinal permeability, resulting in high plasmatic levels of LPS, which may induce low-grade chronic and systemic inflammatory response (Teixeira 2012). Moreover, low doses of LPS are related with a reduced expression of proteins involved in reverse cholesterol transport as ABCA1/ABCG1 and SR-B1 in murine macrophages (Maitra 2013). SCFA and LPS induce, in a coordinate way, the activation of Toll Like Receptors 2 and 4 (TLR2-4) of macrophages infiltrating the adipose tissue, increasing the inflammatory response (Nguyen 2007). Furthermore the activation of macrophage TLR2 and 4 by bacterial LPS suppresses the activity of Liver X Receptors (LXR), involved in the transcription of ABCA1/G1 transporters, both implicated in macrophages Reverse Cholesterol Transport (Lee-Rueckert 2013).

Unlike bacterial LPS, a product of cyanidin-3-O-β-glucoside (α-G) metabolized by intestinal flora has an atheroprotective functions. Protocatechuic acid (PCA) has been showed to have a profound anti-atherogenic effect (Wang 2010). PCA induces the expression of ABCA1/G1 since it promotes cholesterol efflux, through down-regulation of microRNA (miR)-10b, a short non-coding RNA gene involved in regulation of ATP Binding Cassettes genes (Hazen 2012). PCA results from metabolism of anthocyanin above mentioned, found mainly in blackberries and bilberries. Animal data (ApoE knockout mice), showed both PCA and Cy-3-G attenuate atherosclerosis and increase formation of bile acids through activation (Chistiakov 2015).

Recently, a proatherogenic role of the gut microbiota in the metabolism of phosphatidylcholine was shown. Microbiota metabolizes choline and phosphatidylcholine to trimethylamine (TMA), afterwards is converted to a proatherogenic compound, trimethylamine-N-oxide (TMAO). High levels of TMAO
in humans, are associated with an increased cardiovascular risk events, and also chronic kidney disease (CKD) (Aron-Wisnewsky 2015). The concentration of TMAO in the blood increases after consuming foods containing L-carnitine, which is mostly abundant in red meat. It has been discovered that the gut microbiota–dependent metabolism of L-carnitine to produce TMAO accelerates atherosclerosis in ApoE knockout mice through changes in microbial composition and increased colon production of TMA and TMAO (Koeth 2013). Indeed the production of TMAO is dependent on variability of the gut microbiota species, as the bacterial strain of Prevotella was found to produce more TMAO than Bacteroides (Koeth 2013). Functional studies showed that TMAO inhibited reverse cholesterol transport (RCT) and promoted accumulation of cholesterol in macrophages through increasing cell surface expression of pro-atherogenic scavenger receptors (SRs) CD36 and SRA, reducing synthesis of bile acids from cholesterol, and decreasing expression of bile acid transporters in the liver (Chistiakov 2015).

**Figure 7:** Atheroprotective and Proatherogenic effects of microbiota in the host (Chistiakov 2015)
4. Use of Plant Products and Bacteria as Nutraceutical Approach to Atherosclerotic Diseases

In recent years new ways to treat diseases is spreading, aimed to support the medical therapy or replace it. Patients’ non-adherence to medical and medication recommendations is a major public health problem. World Health Organization (WHO) has estimated that 38% of patients on short term treatment and 43% of patients on long term treatment do not adhere until the end of medical therapy. Social, economic and in particular medical factors have been demonstrated to affect therapy adherence also called compliance. Compliance was defined more than 2 decades ago by WHO as the extent to which a person’s behaviour, taking medication, following a diet, and/or executing lifestyle changes, corresponds with agreed recommendations from a health care provider (Ngoh 2009).

non-adherence to medical therapy are due to many reasons, as:

- complex therapies, including co-administration of various drugs, often high-priced drugs;
- lack of patient confidence in the therapy, inasmuch they are not involved in the medical decisions;
- various side effects, especially in chronic therapies;
- ancient patients’ thought is that drugs induce durable and fast improvements, which is not always true, in particular in chronic diseases.

In order to avoid the abandonments of therapies, doctors may involve patients in the choices concerning therapies, showing their benefits, duration and side effects of drugs, so as to empower them. Moreover it may be possible, mostly for chronic therapies, the use of nutraceutical therapies which support pharmacological drugs or in some cases, replace them.

The term "nutraceutical" resulted from the union of words "nutrition" and "pharmaceutical"; it was coined in 1989 by Stephen De Felice which defined nutraceutical as a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of diseases (Kalra 2003).
Nutraceutical therapies include molecular complexes and microorganisms, also called probiotics, which come from plants, milk and functional foods; today the term nutraceutical is often used interchangeably with functional food. According to European Union, a food may be considered as functional whether it affects beneficially one or more target functions in the body in addition to nutritional effects, sufficient to improve health state, well-being and a reduction of risk of disease (Shiby 2013). During last decades nutraceutical field is expanding, mainly due to a wide use of plants metabolites and probiotics bacteria with the purposed to support pharmacological therapies or include them in a healthy diet.

4.1 Probiotics Bacteria for Human Uses: Overview on Origin, Dosage, Uses and Safety

The Food and Agriculture Organization (FAO) and the WHO define probiotics as “live microorganisms which confer a health benefit on the host, when administered in adequate amounts” (FAO 2001). In addition, the International Scientific Association for Probiotics and Prebiotics (ISAPP), a scientific organization dedicated to advancing the science of probiotics claims that the term probiotic is improperly used both commercially, where it labels products with no substantiation of human health benefits, both scientifically, where it has been used to describe bacterial components, dead bacteria or bacteria with health effects in humans (LeBlanc 2014). In order to clarify the probiotic concept, ISAPP claims that bacterial strain may be considered probiotic if:

- it is alive when administered;
- it has undergone all evaluations to document health benefits in the target host;
- it is a taxonomically defined microbe or combination of microbes (genus, species and strain level);
- it must be safe for its intended use.

In order to respect the safety reasons and to demonstrate that health effects are imputable of administered bacteria, it is necessary to genotype the species using either the method of “DNA nucleotide sequencing codifying the subunit 16S RNA”,...
or make phenotypic test for the strain characterization, which occurs analysing bacterial chromosome via Pulsed Field Gel Electrophoresis (PFGE). Indeed, as literature suggests, various evidences indicate that different strains of same species may have different and sometime opposite effects in the host (Aureli 2010). This is confirmed by FAO which claims that “data obtained with one specific probiotic food cannot be extrapolated to other foods containing that particular probiotic strain or to other probiotic microorganisms” (FAO 2001).

4.1.1 Origin

The term probiotic comes from Greek, meaning “for life”. The original observation of healthy effects in the host by some selected bacteria is attributed to the Nobel Prize Eli Metchnikoff, which suggested in 1910 that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes". Metchnikoff observed the dietary habits of ancient group of people (more than 100 years old) of Bulgaria and Caucasus, which drink considerable amounts of acid milk and were in good health. He examined the composition of acid milk discovering a particular bacterium: the Lactobacillus Bulgaricus, which counteract the ageing toxins produced by intestinal microbiota (Mackowiak 2013). In the same period Henry Tissier, a French paediatrician, observed into the faeces of children with diarrhoea, the low presence of bacteria characterized by a peculiar, Y-shaped morphology. These “bifid” bacteria were, on the contrary, abundant in healthy children. Therefore he suggested that these bacteria could be administered to patients with diarrhoea to help restore a healthy gut flora (FAO 2001).

The works of Metchnikoff and Tissier were the first scientific suggestions concerning the probiotic use of bacteria, even if the word "probiotic" was not coined until 1965, it used to name substances produced by microorganisms which promoted the growth of other microorganisms and induce benefit to the host. The veterinaries Lilly and Stillwell began to classify various microorganisms with probiotic capacities. In recent years a wide number of strains has been identified, more than 56 species of lactobacilli and 29 of bifidobacteria, and these numbers are going to increase (Caramia 2004).
4.1.2 Dosage, Safety and Adverse Events

In literature, there are few data about the administered dose of probiotics to obtain a health benefit in the host (humans or animals). The choice of optimal dose depends from the strain(s) used and from the type of required benefit(s); the amount administered is crucial to obtain the benefits through probiotic growth and/or activity in the human body (Morelli 2000).

In order to identify the accurate dose-response, the Agence Francaise de Securite Sanitaire des Aliments (AFSSA) in 2005 first claimed that probiotic concentrations must be greater than or equal to $10^6$ Colony Forming Unit (CFU)/mL in the small intestine (ileum) and at least $10^8$ CFU/g in the colon. The concentrations in the small intestine have been proposed because these concentrations are associated with clinical effects (diarrhoea) in subjects with chronic bacterial infections, whereas the concentrations in the colon have been proposed because they correspond to less than 1/1000 of the autochthonous flora present, which it could be reasonably expected has more chance of being active than flora present at even lower levels (AFSSA 2005).

AFSSA assessments are supported by a lot of in-depth studies about hypocholesterolemic effects of probiotics. Despite this, an accurate dosage of administration has yet to be established. Indeed there is a lack of dosage-response studies to determine the ‘minimal effective dosage’ of probiotics needed to reduce blood cholesterol levels (Ooi 2010).

Various data present in literature showed that probiotics dosages are variable and are dependent on the strains used and the clinical characteristics of subjects. The values of concentration range from $10^7$ to $10^{11}$ CFU/day in humans (Naruszewicz 2002) and $10^7$ to $10^9$ CFU/day in animals (Lubbadeh 1999), although some probiotics are efficacious at lower levels, while some require a substantially higher amount to exert a hypocholesterolemic effect. Indeed, as Naruszewicz et al. showed, the administration of $5.0 \times 10^7$ CFU/mL of *L. plantarum* 299v was sufficient to reduce LDL-cholesterol by 12% (Naruszewicz 2002). In contrast, the consumption of *Lactobacillus acidophilus DDS-I* and *Bifidobacterium longum* in capsules, (3x$10^9$ CFU/capsule daily) did not produce significant changes in lipid profiles (Greany 2004). This suggests that higher dosage may not necessarily be correlated with positive modulation of cholesterol plasma levels; in addition different strains need
varying dosage to exhibit hypocholesterolemic effects. Clinically effective dosages of probiotics should only be established based on studies of the specific strains conducted in humans (Ooi 2010).

Furthermore the biological matrix used for the probiotic administration may influence the effects of administered probiotics. Indeed various bacteria, which are potential probiotics, are not able to survive into the intestinal or gastric environments, because of pancreatic secretions or the low pH of stomach or proximal part of intestine. To address mentioned issues, probiotics are administer through low viscosity vectors to allow a faster passage across the stomach or through the use of micro-encapsulation systems (Caramia 2004; Iravani 2015). Another problem is the genetic and taxonomic similarity of probiotics with strains resident in the gut, because they may be destroyed by immunity system, to restore the microbial equilibrium; in addition, probiotic bacteria are less adapt for the intestinal environment decreasing theirs possibilities to survive into the intestinal lumen, compared with autochthone bacteria. Furthermore several researcher teams have found in the faeces of patients, bacteriophages probiotics strain-specific, which demonstrate the immune response of human against not autochthone strains (Ventura 2011).

In order to solve these problems, new method based on rotation administration of various strains is described, using similar strains with different immune sensibility. This induces the production of specific bacteriophages in the humans against individual strains, which not act against others administered strains. The procedure consists of three parts:

- administration of probiotic strain, inducing the bacteriophages creation by human immunity system;
- administration of strains mixture with different immune sensibility, afterwards immunity system will produce bacteriophages against the strains of mixture, instead of producing ones against probiotic;
- Finally, the new administration of initial bacteria allows the carrying out of probiotic’s healthy effects, since there are not specific bacteriophages against probiotic bacteria within the intestinal environment.
Using this procedure, the level of bacteriophages production is kept low, because human immunity system needs a certain period of time to produce the them (Sozzi 2006).

In terms of probiotics’ safety, the bacteria must be safe when consumed by the healthy population and also when the consumers are severely ill or immune-compromised patients, such as very low birth weight infants, patients with chronic inflammatory bowel diseases, intensive care unit patients, and patients with acute diarrhoea (Sanders 2010; Hill 2010).

Historically, lactobacilli and bifidobacteria species associated with food are considered to be safe, because they are normal commensals of the mammalian flora, as described in previous chapter (Sanders 2014).

However, probiotics may theoretically be responsible for four types of side-effects:

- systemic infections;
- deleterious metabolic activities;
- excessive immune stimulation in susceptible individuals;
- genes transfer which induces antibiotic resistances.

Documented correlations between systemic infections and consumption of probiotic products available in the market, are few and all occurred in unhealthy patients. As reported in literature, thirteen cases of Saccharomyces fungemia (presence of fungi or yeasts in the blood) due to vascular catheter contamination and Bacillus infections linked to probiotic consumption has been registered (Hennequin 2000; Spinosa 2000). No cases of infections from Bifidobacterium have been reported, whereas Enterococcus is emerging as an important cause of nosocomial infections and in addition it induces an increasingly vancomycin resistant (FAO 2002).

The antibiotic resistance may be related with the plasmid transfer in horizontal transmission from one bacterial cell to another one. Plasmid is a double strains DNA molecule containing genes involved in non-essential functions, mainly used by prokaryotes to genetic transfer (Aureli 2010). In 2001, FAO in the guidelines regarding uses of probiotics claims that the bacteria, containing transmissible drug resistance genes, should not be used in foods although in the same report, the FAO is aware that plasmids exist in the most common probiotic strains as lactobacilli and bifidobacteria (especially in strains isolated from the intestine) (FAO 2001); in order
to avoid the mentioned risk, the Scientific Committee on Animal Nutrition of European Union (SCAN) and the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of European Food Safety Agency (EFSA) suggest further research relating to the antibiotic resistance and also they claim that probiotics, which are used in patients, should not harbour transmissible drug resistance genes encoding resistance to clinically used drugs (EFSA 2005).

In order to assess the safety, even among a group of bacteria that is Generally Recognized as Safe (GRAS), EFSA stated that probiotic strains must be characterized at a minimum by different in vitro in vivo tests:

- Determination of antibiotic resistance patterns,
- Assessment of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation),
- Assessment of toxin production, if the strain belongs to a species that is a known toxin producer,
- If the strain belongs to a species with known hemolytic potential, determination of hemolytic activity is required,
- Assessment of lack of infectivity by a probiotic strain in immunocompromized animals would add a measure of confidence in the safety of the probiotic.

Information acquired to clinical trials and epidemiological studies shows that lactobacilli have a long history of use as probiotics without established risk to humans, and this remains the best evidence of their safety (Naidu 1999). Although no relevant pathogenic or virulence properties have been found for lactobacilli, bifidobacteria or lactococci (FAO 2001) some lactobacilli strains have been associated with rare cases of bacteremia, that is the presence of bacteria into the blood (Saxelin 1996). However, an epidemiological study of lactobacilli has shown that there is not increased incidence or frequency of bacteremia related with probiotic lactobacilli amount (Salminen 2002).

In conclusions, in order to focus on benefits and side-effects of probiotics in humans, clinical trials and epidemiological surveillance of adverse incidents in consumers after introduction in the market are strongly recommended (EFSA 2005; FAO 2002).
4.1.3 Probiotic Bacteria and their Effects on Lipid Profile in the Host

Health benefits induced by probiotic bacteria are very species specific and in some cases strains of same species prompt different benefits in the host; therefore, there is no universal strain that would provide all proposed benefits, not even strains of the same species. The strains *L. Rhamnosus* GG (with trade name of Valio), *Saccharomyces cerevisiae Boulardii* (with trade name of Biocodex), *L. Casei Shirota* (with trade name of Yakult), and *B. Animalis Bb-12* (with trade name of Chr. Hansen) are certainly the most investigated probiotic with demonstrated efficacy against several humans diseases as lactose mal-absorption and various types of diarrhoea, due to rotaviral or Clostridium difficile infections (Sharma 2014).

As these strains demonstrated, in addition to improving gut health, probiotics have also been documented to exert other health-promoting effects as the support of the immune system (Frei 2015), prevention of cancer (Chase 2015), anti-oxidative effects (Songisepp 2004), beneficial effects in arthritis disease (Baharav 2004), reduction of allergic symptoms (Di Gioia 2014) and improvement of vaginal candidiasis in women (Falagas 2006), antihypertensive effects (Yeo 2010). In addition, Probiotics have also been studied for their cholesterol-lowering effects (Pereira 2002).

4.1.3.1 Discovery and Development of Probiotics Bacteria Products as Hypocholesterolemic Agents

Shaper and colleagues (1963) and later Mann (1974) were the first researchers that have dealt with probiotics effects on cholesterol levels in the blood. They observed that men from the tribes of Samburu and Maasai warriors in Africa showed a reduced serum cholesterol after consumption of large amounts of milk fermented with a wild Lactobacillus strain. Afterwards, they affirmed that even Bifidobacteria species have cholesterol lowering effects in the host (Mann 1974).

Since then, the potential hypocholesterolemic effect of fermented milk products containing lactobacilli and/or bifidobacteria has been investigated in in vitro tests and in animals or humans studies as well. Later Harrison and Peat, in 1975, found that cholesterol levels of babies fed with milk containing Lactobacillus acidophilus is
decreased, whereas the number of probiotics in their stools increased (Harrison 1975). In most of these studies (especially the early researches), a decrease in serum cholesterol was only observed in humans during the consumption of very high doses of fermented dairy products. Other investigators using “normal” doses of the fermented milk product failed to confirm such findings (de Roos 1999).

It was suggested that the contradictory results obtained could be, at least in part, related to experimental design (Taylor 1998). Some of the factors addressed were lack of statistical power, use of inadequate sample sizes, failure to control nutrient intake and energy expenditure during the experiments. More recent dietary studies using random double-blind placebo procedures and higher ranges of human subjects have reached the same conclusions (Ooi 2010).

In recent years, probiotic approach to obtain lower lipid levels in the blood is spreading in western World. Indeed various products with proved cholesterol lowering are spreading into the European market, which may support drug therapies or in some cases, replace them. For example, Danone Company launched Actimel Cholesterol Control® in Belgium, containing the suggested cholesterol-lowering probiotic Lactobacillus acidophilus, while the Dutch company Mona introduced the Fysiq®, a cultured dairy-based drink that contains the probiotic Lactobacillus acidophilus (Sharma 2014).

4.1.3.2 Bacterial Strains as Probiotics: Studies on Principal Bacteria Used and their Benefit on Lipid Profile in the Host

Lactobacilli and the Bifidobacteria are the most common strains subjects of various studies aimed to the prevention and treating of CVD.

Lactobacillus acidophilus bacteria (LAB) are Gram-positive microorganisms. They prefer anaerobic conditions and also are aero and acid tolerant. In addition they are strictly fermentative, producing lactic acid as a main product (Stiles 1997). According to their carbohydrate metabolism, LAB are divided into two distinct groups. The homo-fermentative group including Lactococcus, Pediococcus, Enterococcus, Streptococcus which utilize the glycolytic pathway termed Embden–Meyerhof–Parnas to transform a carbon source into lactic acid. Instead, in the heterofermentative group, lactic acid and equal amounts of CO₂, ethanol or acetate as
well, are products via Pentose phosphate route, which is a metabolic pathway parallel to glycolysis that generates NADPH and pentoses (5-carbon sugars). Members of this group include Leuconostoc, Weissella, and some other lactobacilli (Sharma 2014). Bifidobacterium are normal inhabitants of the human GI tract. Bifidobacterium is a Gram-positive, non-motile, often anaerobic bacteria. Recent in vivo scientific studies using animals or human volunteers have shown that consumption of live Bifidobacterium has an effect on the gut microflora. Selected strains survive stomach and intestinal transit and reach the colon in abundant numbers (Schell 2002). As described in literature, there are many studies of cholesterol lowering of Lactobacilli and Bifidobacteria strains. Among the first controlled study, Gilliland et al. has conducted experiments on pigs, fed with high cholesterol diet for 10 days followed by feeding with probiotic strains of *L. acidophilus* P-47 and RP-32, found significant difference in treatments (-11.8% reduction of total cholesterol) (Gilliland 1985). Afterwards, various studies, trials and meta-analyses have occurred. Jones et al. analysed the activity of a yogurt formulation containing microencapsulated *Lactobacillus reuteri* NCIMB 30242, in a total of 114 hypercholesterolaemic subjects in double-blind, placebo-controlled, randomised. Patients have swallowed the yogurt twice per day for 6 weeks, including 2-week of washout. *L. reuteri* NCIMB 30242 achieved significant reductions in LDL-cholesterol (8.92%), total cholesterol (4.81%) non-HDL-cholesterol (6.01%) over placebo, and a significant change in apoB-100 of 20.19 mmol/l as well (Jones 2012). Other researchers focused on bifidobacteria strains as Guardamanga et al. which analysed the effects of a mix composed by three bifidobacteria (*B.analis lactis* MB2409, *B.bifidum* MB109B, and *B.longum longum* BL04) on lipid profile of 38 children affected by primary dyslipidemia. Researchers founded a slight reduction of total cholesterol and LDL, respectively 3.4% and 3.8% after 3 months of treatment (Guardamagna 2014). An important meta-analysis of randomized controlled clinical trials occurred, aimed to evaluate the effect of probiotic *L. bulgaricus, L. sporogenes, E. faecium, B. lactis* e *B. longum* on serum LDL-C and total cholesterol levels. Pooled data from 13 trials of a total of 485 participants with high, borderline high and normal serum cholesterol
levels found that probiotic consumption significantly lowered LDL-C and total cholesterol levels among all categories, compared to the control. Indeed the pooled mean net change in total cholesterol for those treated with probiotics compared to controls was -6.40 mg/dl, than the mean net change in low-density lipoprotein (LDL) cholesterol was -4.90 mg/dl and mean net change in triglycerides was -3.95 mg/dl (Guo 2011).

Probiotics researches are not limited only on the studies of Bifidobacteria and Lactobacilli effects, as the two double-blind, placebo-controlled trial aimed to study the hypocholesterolemic effects of a Danish milk product (with trade name of Gaio). This milk contains Enterococcus faecium (about $2 \times 10^8$ CFU/ml) and two strains of S. thermophilus (about $7 \times 10^8$ CFU/ml). Agerbaek et al. tested 29 normo-cholesterolemic men, all aged 44 years. The treated group exhibited a reduction of 10% in LDL-cholesterol following six weeks of diet supplemented daily with 200 mL of the product, compared with placebo group (Agerbaek 1995).

4.1.3.3 Cholesterol Lowering Mechanism of Probiotics

Probiotics reduce cholesterol absorption of enterocytes by three ways: by assimilating and binding, by enzymatic metabolism and by degradation. Indeed probiotic strains assimilate the cholesterol for their own metabolism or they may get bound to the cholesterol molecule or furthermore they are capable of degrading cholesterol to its catabolic products.

- Cholesterol assimilation into the bacterial cells

It has previously been published that several bifidobacterial strains as the B. bifidum, B. breve, and B. animalis subsp. lactis are able to assimilate cholesterol (Bordoni 2013). This mechanism is based on the capability of Bifidobacteria to incorporate exogenous cholesterol and lipids from intestinal lumen, so as to reduce cholesterol uptake of enterocytes. As literature suggests, in presence of cholesterol, an up-regulation of genes encoding ABC-type carriers is increased. Piemontese et al. founded in Bifidobacterium bifidum PRL2010, a specific mechanism, mediated by ABC transporters for cholesterol intake (Piemontese 2015). The up-regulation of ABC-genes has been observed for other probiotics bacteria as Lactobacillus
acidophilus and Lactobacillus plantarum (Remagni 2013). Cholesterol is predominantly found in the membrane and exerts a crucial role in bacterial membranes, since it interacts with phospholipids modulating the fluidity. This suggests that cholesterol incorporated into the cellular membrane alters the fatty acid composition of the cells, increasing the ratio between total saturated and unsaturated fatty acids, inducing higher cellular resistance and subsequently higher cellular resistance against lysis (Kimoto 2002). In addition, bacteria are also able to assimilate cholesterol during non-replicating phase, as Lactobacillus acidophilus and Lactococcus lactis. In vitro researches demonstrated that these non-growing bacteria may remove cholesterol from enriched medium, through chemical interactions among cholesterol and high molecular weight structures situated onto the cellular surface termed exopolysaccharide, of amino acid and polysaccharide nature (Liong 2005).

**Cholesterol degradation after conversion in coprostanol**

Cholesterol can also be converted in the intestines to coprostanol, which is directly excreted in faeces. 5β-Coprostanol is a 27-carbon stanol formed from the bio-hydrogenation of cholesterol in the gut of higher animals, as humans. This conversion leads to reduction of cholesterol absorbed by enterocytes, decreasing the plasma concentration. Bacterial genera as Arthrobacter, Corynebacterium, Mycobacterium, Nocardia, Pseudomonas, and Rhodococcus, have this enzymatic pathway which includes at least four transformations that require molecular oxygen as a co-substrate. Chiang et al. found that cholesterol dehydrogenase/isomerase produced by bacteria as Sterolibacterium denitrificans was responsible for catalysing the transformation of cholesterol to coprostanol. This pathway of cholesterol degradation is found also in Bifidobacteria strains, as the B. animal and B. longum (Noriega 2006), and in the species of Bifidobacterium bifidum as the subspecies PRL2010 (Piemontese 2015).

As shown in figure 8, the pathway starts with the oxidation of cholesterol to cholest-5-en-3-one (compound S1), which is isomerized to cholest-4-en-3-one (S2), both steps mediated by AcmA (cholesterol dehydrogenase/isomerase), which catalyse the
oxidation of the 3-OH group, followed by $\Delta^5$-to-$\Delta^4$ isomerization. Afterwards, the metabolite may interact with $\text{H}_2\text{O}$ oxidizing to 25+ -hydroxycholesterol-4-en-3-one (S4) or it can originate the cholesta-1,4-dien-3-one (S3) and successively the 25-hydroxycholesta-1,4-dien-3-one(S5), mediated by the enzyme cholest-4-en-3-one-$\Delta^1$ dehydrogenase (AcmB) (Chiang 2008). In addition, in a recent in vitro study, Lye et al. evaluated the conversion in coprostanol of lactobacilli strains as *Lactobacillus acidophilus*, *L. bulgaricus* and *L. casei* ATCC 393 via fluorometric assays. The authors detected both intracellular and extracellular cholesterol reductase in all strains of probiotics examined, indicating possible intracellular and extracellular conversion of cholesterol to coprostanol (Lye 2010).

![Enzymatic pathway which transform cholesterol in coprostanol (Chiang 2008).](image)

- **Bile Salt Hydrolase (BSH) activity**

The Bile Salt Hydrolase is a common enzyme responsible of bile salt deconjugation in the enterohepatic circulation; it has been detected in various indigenous bacteria of human intestine and in various probiotics as Bifidobacteria and Lactobacilli strains. BSH is able to hydrolyse conjugated glycodeoxycholic acid and taurodeoxycholic acid, mostly composed by cholesterol, leading to the deconjugation of glyco- and tauro-bile acids, chief components of bile. BSH may be a potential target because its hydrolyse activity results in a low amount of bile acids in the intestinal lumen, which are replaced by new synthetized molecules, contributing to the reduction of cholesterol serum levels (figure 9) (Jones 2013).
Recently, a randomized, placebo-controlled clinical trial showed the significant reduction of TC and LDL-C resulting by the administration of BSH-active *L. gasseri* CHO-220 combined in a capsule with the prebiotic inulin (Ooi 2010). In addition, it has published a double-blind, randomized study to evaluate the BSH activity of the *L. reuteri* NCIMB 30242, administered as microcapsule (3x $10^9$ CFU/capsule) over 9 weeks. Lipid profile of subjects consuming the capsules attained significant reductions in LDL-C of 11.64%, TC of 9.14%, non-HDL-C of 11.30% and apoB-100 of 8.41%, over the 9-week treatment period, compared to placebo. In order to support the BSH mechanism, Jones et al. found significant increases in deconjugated bile acids, indicating increased intraluminal BSH activity (Jones 2012).

In addition, by modifying the bile acid pool profile, BSH-active bacteria may influence the farnesoid X receptor (FXR), a bile acid nuclear receptor. It has been shown that decreased FXR activity results in an increased catabolism of cholesterol and synthesis of bile acids mediated by CYP7A1 and down-regulation of the nuclear receptor small heterodimer partner (SHP). Latter enzyme repress the other nuclear receptors through its binding to form a non-productive heterodimer (Thomas 2008). The down-regulation of SHP results in the up-regulation of the liver X receptor (LXR), then this leads to the up-regulation ABCG5/G8, promoting the cholesterol biliary excretion. Usman et al. found that *Lactobacillus plantarum* KCTC3928 administration to mice resulted in significant LDL-C and triglyceride lowering effects, increased faecal bile acid excretion, hepatic bile acid synthesis and increased expression of CYP7A1, enhancing cholesterol catabolism and bile synthesis (Usman 2000). Another proposed mechanism of action associated with BSH activity involves the inhibition of NPC1L1. Indeed, a decrease in NPC1L1 activity was demonstrated in rats after administration of *L. acidophilus* (Huang 2010). In addition, in recent research, Yoon et al. have demonstrated a down-regulation of NPC1L1 in Caco-2 colon epithelial cells by *Lactobacillus rhamnosus* and *L. plantarum* (Yoon 2013).
Short chain fatty acids (SCFA) as modulators of cholesterol uptake by enterocytes

After probiotic colonization of human gut, they begin to ferment food-derived indigestible carbohydrates that results in an increased production of a series of metabolites called short-chain fatty acids (SCFA). These molecules are fatty acids with an aliphatic tail of less than six carbon atoms and are particularly important for microbiota health, because they are the primary energy source for colonic cells (Wong 2006). In addition, they have anti-carcinogenic and anti-inflammatory properties (Greer 2011). As described in previous chapter, the most diffuse SCFA, (acetate, propionate, and butyrate) play an important role in cholesterol reduction. Indeed acetate in the serum seems to increase total cholesterol, while propionate increases blood glucose and tends to lower the hypocholesterolemic response caused by acetate, which reduces its utilization by the liver for fatty acid and cholesterol synthesis (St-Onge 2000).
SCFAs have been reported to activate the enzyme peroxisome proliferator-activated receptors (PPARγ), which induces the angiopoietin-like protein 4 (ANGPTL4), a LPL inhibitor. The inhibition of LPL reduces the storage of VLDL and chylomicron triglycerides in adipose tissues, and regulates fatty acid oxidation in muscle and adipocytes (Korecka 2013).

4.1.3.4 Faecal Microbiota Transplantation (FMT): Potential Therapy against Atherosclerosis Diseases

A new approach to modify the microbiota and obtain healthy benefits for the host is the faecal microbiota transplantation (FMT). The first known experiences of human donor faces as a therapeutic agent occurred in antique China (fourth century). More recently, the researcher Lewin assess that “consumption of fresh, warm camel faces has been recommended by Bedouins as a remedy for bacterial dysentery; its efficacy was confirmed by German soldiers in Africa during World War II” (Lewin 1999). First Eiseman and co-worker (1958) used FMT in mainstream medicine for the treatment of pseudomemb ranous colitis. Afterwards the popularity of FMT increased, due to its efficacy and ease of use for the treatment of patients with Clostridium difficile Infection (CDI) (Eiseman 1958). In a recent and systematic review of 317 patients with CDI treated with FMT, a slight rate of disease resolution it has been found among recipients of stools from related donors (93%) than unrelated donors (84%). By sex, IMT from a male donor showed 86% resolution, with no relapses, and IMT from a female donor showed 100% resolution but 8% relapse (Gough 2011).

FMT consists in the administration of a faecal solution from a donor into the intestinal tract of a recipient. According to the study conducted by Academic Medical Center of Amsterdam, the healthy donors must be a family member or a friend of recipient. Donor’ stools (200–300 g) have to be dissolved in 500 mL sterile saline solution and used preferably within 6 hours of passage (van Nood 2013), although in another study, frozen stool samples were used in FMT for CDI, reporting comparable results to those of studies that used fresh faecal samples (Hamilton 2012). As literature suggests, there are various and heterogenic studies
about the preparation of material for FMT. However, the lack of sufficient numbers and controls in most studies makes difficult to draw solid conclusions. The faecal material for transplantation can be infused by various methods, as nasogastric or nose-jejunal tube, colonoscopy or enema. Gough et al in a review, suggested that FMT via esophagogastrroduodenoscopy, nasogastric tube, or nose-duodenal tube resolved CDI in 76% to 79% of patients compared with the mean worldwide rates of 91% for FMT via colonoscopy (Gough 2011).

To date, FMT appears to be safe; indeed various researcher teams have performed more than 3200 faecal transplantation procedures, do not revealing any serious adverse events. Most patients, after treatment, report diarrhoea on the day of infusion, and a small percentage of them have abdominal cramping or constipation. Serious adverse events were reported for only 3 of 317 patients (peritonitis, or enteritis). In another case report, nasoduodenal FMT for Crohn’s disease resulted in transient adverse effects, including fever and abdominal tenderness in 3 of 4 patients. However, these effects disappeared for all patients over the following 2 days (van Nood 2013; Borody 2012).

As mentioned in literature, most clinical experience has focused on FMT in patients with CDI or with inflammatory bowel disease (IBD) complicated by CDI (Reddy 2013). In addition, it has been suggested that FMT is useful against cardio-metabolic diseases. Indeed obese patients or with cardiovascular diseases have a poorer microbiota population compared with healthy people. The scarcity of microbiota population in these patients induces an increase of intestinal permeability, which would lead to metabolic endotoxemia (presence of endotoxins in the blood), bacteremia and chronic inflammation. Furthermore, in obese subjects intestinal permeability induces an increased plasma levels of bacteria or their proteins (mainly lipopolysaccharide or endotoxin) (Kootte 2012). In order to avoid and to limit all these consequences, the FMT could rebalance the normal gut flora, introducing bacterial strains from healthy people, who are able to execute normal functions of microbiota mentioned in previous chapters, as the production of SCFA (Smits 2013).
4.2 Consumption of Plants Products as Potential Strategies against Atherosclerosis Diseases

During the recent years, researchers have studied in depth the activities of molecules coming from the plants and theirs effects in the humans. These compounds may avoid or limit the adverse effects of diseases, administering them with the pharmacological therapies or including them in a controlled diet.

As literature suggests, the wide class of polyphenols possesses well-documented, beneficial anti-inflammatory, antioxidant mechanisms and atheroprotective effects, demonstrated in vitro, in animals and in humans.

As probiotic bacteria, plant products may be included in nutraceutical strategies aimed to support pharmacological therapies, or in some cases replace them in order to avoid side effects of drugs. Nutraceutical strategies include the administration of compounds extracted by the plants or their active metabolite in the form of pills, beverages or others drug vehicles; other way is the controlled diet, where the patient takes precise amounts of nutritional foods, containing molecules of plants origin with beneficial effects on human health.

4.2.1 Polyphenols: Origin, Classification and their Metabolism in the Humans

Polyphenols are the widest and heterogeneous group of plant metabolites, mostly presents in foods and beverages (Perez-Jimenez 2010). Polyphenol are produced by several biosynthetic pathways in plants and they support vital functions as structure, pigmentation, pollination, pathogen and predator resistance, growth and development (Crozier 2007). These important functions for the survival of plants account for wide array of biological activities. In the past these metabolites have been considered anti-nutritional food constituents and only afterwards it has been assessed that they may be potential healthy compounds, when introduced in the human diet in proper amounts. In the attempt to investigate the potential activities of polyphenols, in recent years many clinical trials and epidemiological studies have been performed on
the potential role of polyphenols in several diseases as atherosclerosis. Furthermore, several in vitro analysis have recently tried to investigate the mechanisms of action (Del Rio 2013).

The benefits for human health of polyphenols and their mode of actions are emerged through various in vitro, in vivo researches and clinical trials in humans. According to several studies, the effects of polyphenols in the host are due to their transformation which occurs in various parts of body, following the typical detoxification pathway common to xenobiotic and drugs. Indeed after ingestion, the transformations starting with the activities of the oral saliva and gastric secretion to modify the native polyphenolic structures (Walle 2005). Afterwards, in the small intestine, a limited part of conjugated forms which is unmodified in the oral cavity, cross the enteric barrier reaching the portal circulation. Two different pathways of absorption exist, former route is mediated by the lactase phlorizin hydrolase, an enzyme present in the brush-border of the small intestine epithelial cells (Day 2015); alternative route is an the catalysis by a cytosolic β-glucosidase, which seems able to operate after the intake of polyphenols into the cells due to the active sodium-dependent glucose transporter, SGLT-1 (Gee 2000).

Once polyphenols passed the enteric barrier, they undergo phase II enzymatic detoxification through conjugation with chemical groups such as glucuronic acid, sulphate, and methyl groups operated by the action of uridine 5’-diphospho-glucuronosyltransferases, sulphotransferases and catechol-O-methyltransferase respectively. This conjugation step first occurs before entering into the blood stream and in the liver as well. Furthermore, in this organ, the metabolites are modified by the phase II enzymes in more hydrosoluble compounds, then they are transferred to the systemic circulation, or to enterohepatic re-circulation, and are finally excreted. However, it has been estimated that only a limited part of the ingested polyphenols (5–10%) is actually absorbed, in the small intestine. The remaining unmodified polyphenols (90–95%) may accumulate in the large intestine at high concentrations, where they are exposed to the enzymatic activities of microbiota. Actually, the gut microbiota transform the polyphenols in a large number of low-molecular-weight
compounds, which could be more absorbable and in some cases have beneficial functions in the host (Palafox-Carlos 2011).

4.2.1.1 Classification and Metabolism in the Humans

Polyphenols in plants are available as oligomers and polymers, or esterified with other chemical compounds (sugars or organic acids) whereas rarely are in form of aglycone. According to their physiological role in a plant and to the structure, they can be found in the aqueous content of the vacuole, or more or less tightly joined to the polysaccharidic constituents of the cell wall. In order to identify all molecules of wide class of polyphenols, they are commonly classified from chemical viewpoint. Polyphenols is a heterogeneous class, characterized by a core of at least one aromatic ring, linked with other phenolic-, hydroxyl-, carbon- or other chemical groups. This group includes an extensive variety of molecular structures with different molecular weights and with different values of solubility and membrane permeability. According to their structures, polyphenols are generally classified into flavonoids and non-flavonoids. The first group comprises several subclasses, including flavonols, flavanones, flavones, isoflavones, anthocyanins, and flavan-3-ols, whereas non-flavonoids group includes mainly tannins, stilbenes, phenolic acids, and hydroxycinnamates (Crozier 2009).

Principal subclasses of flavonoids group are:

- **Flavonols**

Flavonols are the most common flavonoid of plant kingdom, and therefore with a high dietary intake per day (50 mg). Indeed, high amounts of Flavonols are widely diffuse in vegetables and fruits as kale, onion, broccoli, tomato, and berries, with values from 1200 mg/kg of onion to 40 mg/kg of apples (Appeldoorn 2009). Quercetin is the most widespread flavonol, followed by kaempferol,isorhamnetin and myricetin. Its glycosidic form is abundant in onions, apples, tea, broccoli and red wine. In the human intestine, the aglyconic form of ingested quercetin is cleaved in small intestine, afterward converted in glucuronide, sulphate, and methylated metabolites by the enterocytes and by liver. The remnant fraction of quercetin is
metabolized by colonic microbiota. Here, the enzymatic activities of bacteria lead to the breakage of the flavonol skeleton, releasing several polar metabolites with low molecular weight as 3′-4′-dihydroxy-phenylacetic acid. The great variability of metabolites depending on different bacterial population present in the human gut of each person, whereby diverse bacteria perform different metabolites (Mullen 2006).

- **Anthocyanins**

Anthocyanins are the natural pigments responsible for the red-blue colour of many fruits; the dietary intake is varies greatly, from 60 mg to 1 gr per day (Clifford 2000). The main dietary sources of anthocyanins are berries (up to 5000 mg/kg), red grapes (up to 7500 mg/kg) and red wine (up to 350 mg/L) (Mena 2011). Anthocyanin are composed by anthocyanidin (the aglyconic part of molecule), as cyanidin, pelargonidin, peonidin, delphinidin, petunidin, and malvidin, most common in the plants, and the sugar part which bound to anthocyanidins, as glucose, galactose, rhamnose, and arabidose (Bridle 1997). Anthocyanins appear to have low bioavailability (<1%), because of the wide metabolism occurring in large intestine by microbiota. Once in the large intestine, anthocyanins are hydrolysed by the local microbiota through to β-glucosidase activity, resulting a broad array of phenolic and aldehydic constituents as Protocatechuic, gallic, syringic, and vanillic acid (Vitaglione 2007). In addition their phase II conjugates (methyl, glucuronide, and sulphate), rapidly reach maximum plasma concentrations which is into the nM range (McGhie 2007).

- **Flavanones**

Flavanones are a sub-group of flavonoids mainly present in citrus fruits and tomatoes. These compounds have the daily intake from 7.6 to 93.7 mg per day (Mink 2007). Most common flavanones absorbed in the colon are the naringenin (common in the grapefruit and sour orange), the hesperetin (common in the orange) and the flavanone glycoside as hespere-tin-7-O-rutinoside. Both glucuronide and sulphoglucuronide metabolites of hesperetin and naringenin, although are absent in
plasma, were recovered in urine, emphasizing substantial post-absorption phase II metabolism (Gordon 2012).

**- Flavan-3-ols and proanthocyanidins**

Flavan-3-ols and proanthocyanidins are the most largely consumed molecules by Western population, because they are widely common in fruits and vegetable as the tea, cocoa and dark chocolate, apples, pears, grapes, berries and red wine. Flavan-3-ols are a complex subclass of polyphenolic substances without glycoside residues. Monomeric flavan-3-ols include catechin, epicatechin, gallocatechin, epigallocatechin and their galloyl substituted derivatives (epicatechin-gallate and epigallocatechin-gallate). Proanthocyanidins, also called tannins, are a polymer of flavan-3-ol monomeric units and their actual dietary intake has not been adequately evaluated, but is estimated to be around 300 mg per day. According to the structures of the monomer, subclass of tannins is composed by procyanidins (polymer of epicatechin) and prodelphinidins (polymer of epigallocatechin) (Santos-Buelga 2000).

The weight, solubility and the bioavailability of these subclasses is very heterogeneous. However, it has been estimated that only about 8–17% of dietary flavan-3-ols are bio accessible in the small intestine, while the remaining unabsorbed fraction of flavan-3-ol monomers and proanthocyanidins are metabolized in the colon by microbiota, to obtain several low molecular weight metabolites, namely phenylpropionic, phenylacetic, hippuric, and benzoic acids (Stoupi 2010).

**- Hydrolysable tannins: gallotannins and ellagitannins**

Hydrolysable tannins are the main group of plant tannins, with more than 500 structures identified. They are composed of polyesters of sugars (usually glucose) and phenolic acids. Hydrolysable tannins are divided in two subclasses: gallotannins which include the gallic acid, and ellagitannins, that spontaneously rearranges into ellagic acid upon hydrolysis (Bakkalbasi 2009).
Ellagitannins are typical constituents of many plant families, whereas the distribution of gallotannins in nature is rather limited. However, former compounds are common in few fruits and nuts, including raspberries (up to 2600 mg/kg), strawberries, blackberries, blueberries, pomegranate (up to 5700 mg/l in juice), muscadine grapes, and persimmon, as well as walnuts (up to 590 mg/kg) (Landete 2011). The bitter and astringent taste of hydrolysable tannins is the reason of their presence in tissues with low levels of nutrients (barks, wood). After ingestion they are hydrolysed to yield gallic acid and/or ellagic acid. Afterwards, they may be metabolized in pyrogallol and pyrocatechol by the gut microbiota or may be absorbed and appear in the circulatory system free or methylated (3-O-methylgallic acid, 4-O-methylgallic acid, 3,4-di-O-methylgallic acid). Free ellagic acid can be absorbed and it is subject of an extensive phase II metabolism; however mainly (more than 99%) is metabolized by microbiota and transformed in urolithins (González-Barrio 2010). Urolithins are microbial metabolites possessing a 6H-dibenzo-[b,d]pyran-6-one structure with different phenolic hydroxylation patterns. It has been recognized the urolithin D, urolithin C, urolithin A, and urolithin B. It must be noticed that in the circulatory system ellagic acid is not detected, because is totally metabolized in urolithins, which appear in the circulatory system almost exclusively as glucuronide, sulphate and methylated metabolites (García-Muñoz 2014).

Principal subclasses of non-flavonoids group are:

- **Phenolic acids**

Phenolic acids are the most abundant non-flavonoidic component of diet, with an intake of ~600 mg per day. Principal polyphenols are hydroxybenzoic and hydroxycinnamic acids; former is widespread in red wine, tea, and some berries and nuts, latter is common in coffee, apples, tea, berries, green vegetables and grain. Gallic acid is the most relevant hydroxybenzoic acid and is widely present in the form of complex sugar esters (gallotannins) and in non-sugar galloyl esters (Crozier 2009). Among hydroxycinnamic acids, the most representative compounds are caffeic, ferulic and p-coumaric acids. These compounds are named chlorogenic acids, as caffeoylquinic acids, when are esterified with quinic acid, feruloylquinic
acids and p-coumaroyl-quinic acids. Coffee is one of the major dietary sources of chlorogenic acids (1750 mg/l), together with apples (600 mg/kg), and many green vegetables (aubergines, 660 mg/kg). Ferulic acid is the most abundant hydroxycinnamate found in cereal grains, which constitute its main food source. In humans the caffeoylquinic acids is converted by microbiota in caffeic acid and dihydrocaffeic acid, which is further metabolized to dihydroisoferulic acid free and sulphated dihydrocaffeic, which are present in the blood (Stalmach 2009).

- **Stilbenes**

Stilbens are less widespread in plant foods compared to other classes of polyphenols. They do not have particular dietary relevance, except for the resveratrol (3,5,4’-trihydroxystilbene), which is diffuse in red wine, grapes, peanuts, pistachios, and berries (Zamora-Ros 2010) Resveratrol and all the conjugated derivates (trans-resveratrol-3-O-glucoside) have low bioavailability; indeed they are rapidly absorbed and metabolized in humans. Resveratrol is absorbed by enterocytes in the upper gastrointestinal tract and can be modified and hepatocytes leading to the production of the glucuronide and sulphate forms. In addition, the intestinal bacteria are able to convert resveratrol into dihydroresveratrol, which, at least partially, is absorbed and further metabolized to conjugated forms that can be excreted in urine. The overall scenario regarding the actual properties of these substances is far from complete, and the scientific knowledge on the bioactivity of resveratrol metabolites is limited, with conflicting results (Chachay 2011).

### 4.2.2 Plant Products Activities against Atherosclerosis and Cardiovascular Disease

Several epidemiological observations have linked polyphenolic intake to atheroprotective effects in humans. As literature suggests, various in vivo, in vitro studies and clinical trials have shown a correlation among improvement of lipid metabolism and the consumption of molecules coming from nuts, coffee, cocoa,
grapes, and berries or after controlled diet (Zanotti 2015). Vegetables and fruits are the principal sources of antioxidant vitamins and various phytochemical as polyphenols. Consequently, to study their beneficial effects it is essential to examine the food which contain that molecules. The relationships between eating vegetables and fruits and preventing CAD and stroke have been demonstrated in many epidemiological studies. Liu et al. assessed that the associations between vegetable and fruit consumptions and risk of cardiovascular diseases. The relative risk between those with the lowest vegetable and fruit consumption (median value: 2.6 servings/day) and those with the highest consumption (median value: 10.2 servings/day) was 1.0 and 0.68 respectively (Dauchet 2006). In addition, Joshipura et al. has performed a study among almost 42,000 men and 84,000 women also demonstrated a relative risk for CAD of 0.80 in the highest quintile of vegetable and fruit consumption. In their study, the consumption of green leafy vegetables and vitamin C-rich fruits and vegetables contributed most to the protective effect of vegetables and fruits (Joshipura 2009). Also the Mediterranean diet may be seen as a nutraceutical approach to control and reduce CVD. Indeed its components have various effect on humans, as assessed a trial of primary prevention (PREDIMED). In this trial the participants have assumed Mediterranean diet supplemented with nuts, showing a significant reductions of waist circumference (mean -5 cm), of concentrations of medium-small (<27 nmol/l) and very small LDL (<111 nmol/l); in addition a decreased LDL particle number (<98 nmol/l) and an increase of large LDL concentrations (54 nmol/l) have been observed (Damasceno 2013). These effects are due to several components of nuts which play a crucial role, in addition to phenolic substances, as γ-tocopherol, α-linolenic acid, linoleic acid, phytosterols (Kris-Etherton 2008). Specifically, decreased LDL is accompanied by a shift towards increased particle size and a reduction in the most atherogenic small, dense LDL. Importantly, the effects of nut intake are dose-related and are likely to be independent of nut types; almonds, hazelnuts, pecans, pistachios, walnuts, peanuts are all effective, with a reduction in the total cholesterol by 5% and LDL concentration by 10%, as well as an increase in the HDL/LDL ratio by 8.3% (Damasceno 2011).
The flavanone Naringenin, is another example of metabolite which influences lipid profile. This molecule, predominant in grape fruit may modulate in vivo VLDL assembly through the inhibition of ACAT2, apoB secretion and MTP activity. Moreover, naringenin may cause a reduction in the cholesterol level by inhibiting HMG-CoA reductase, in addition, it may induces the expression of LDL receptor and ApoAI production operating on PPARα, PPARγ and LXRα, leading to the induction of the apoAI gene. It has been demonstrated that the metabolic activities of gut microbiota are important for the polyphenols to exert their anti-atherogenic effects. Wang and co-workers showed that the lipid profile of mice is influenced by protocatechuic acid, a metabolite of cyanidin-3-O-β-glucoside, conversely the administration of the latter molecule does not have effects in mice (Jung 2006).

It was also documented that olive-oil consumption positively affects lipid profiles. Many components can be responsible for this activity, including unsaturated fatty acids, simple phenols, triterpenic acids and phytosterols. Evidences of specific benefits associated with olive oil-derived polyphenols come from several studies, demonstrating that olive oil provides protection against different risk factors for coronary heart disease. The results of a meta-analysis of 14 studies showed that replacement of Saturated Fatty Acids (SFA) by oils enriched in Mono Unsaturated Fatty Acids (MUFA) versus Poly- Unsaturated Fatty Acids (PUFA) had similar effects on total, LDL, and HDL cholesterol. The PUFA-enriched oil had a slight triglyceride lowering effect, and there was an increase in HDL cholesterol after MUFA consumption in some studies (Gardner 1995). In addition, the intake of olive oil is associated with an un-regulation of genes involved in cholesterol efflux, as ABCA1, SR-BI and PPARs genes (Konstantinidou 2009).

Another food widespread in western world are the grapes and all products originate from them as wine, grape juice, grape-based extracts from grape seeds and grape pomace. It is well established that moderate amount of red wine produces cardio protective effects (Chiva-Blanch 2013). Many studies have demonstrated that all non-alcoholic grape products may produce atheroprotective effects (Dohadwala 2009). Grapes, especially the red varieties, contain significant amounts of anthocyanins, flavan-3-ol monomers, proanthocyanidins, phenolic acids
hydroxycinnamates, resveratrol and flavonols. The ingestion of grape products has been associated with a decrease in the amount of LDL (−17%), triglycerides (−15%) and apoB, and an increase in the amount of HDL (+12%) and ApoAI, in both healthy subjects and in patients with high cardiovascular risk (Vislocky 2010). Most important polyphenol in grapes is the resveratrol. Resveratrol has been demonstrated to reduce apoB48 and apoB100 production in the liver and intestine of obese individuals (Dash 2013). In ApoE/- mice, resveratrol and a resveratrol-containing mixture have been shown to reduce atherosclerotic lesions. Indeed it reduces total and LDL cholesterol and increases HDL cholesterol in the ApoE/- mice, feed with a 0.02% or 0.06% (w/w) resveratrol supplemented in chow diet for 20 weeks (Do 2008). Whereas no effects on plasma lipid levels are observed in the hypercholesterolemic rabbits fed with resveratrol daily dose 3 mg/kg bodyweight for 12 weeks. Resveratrol may influences lipid profile with several supposed mechanisms, as the stimulation of NO production (Khandelwal 2010), the inhibition of LDL oxidation (Belguendouz 1997), the reduction of vascular inflammation and prevention of leukocyte adhesion and finally inhibition of smooth muscle cell proliferation (Kim 2010). An important compound mostly present in the wine is the quercetin-3-O-glucuronide. Although the mechanisms of action of this molecule, underlying beneficial effects in humans, is not completely elucidated, some hypotheses can be proposed. Some researches highlight the quercetin-3-O-glucuronide influence the lipid metabolism in cultured macrophages; likely enhancing ABCA1 expression through a LXR-mediated mechanism with a potentially positive impact on the cell cholesterol efflux. It has also been indicated to inhibit CD36 expression, thus preventing foam-cell formation (Ohara 2013).

The coffee is the widely consumed beverage in the world. It contains a complex mixture of substances, in which both beneficial and detrimental compounds coexist. Indeed, it is well known that caffeine induces cardiac dysrhythmias and increases blood pressure, whereas the diterpenes, as cafestol raise plasma cholesterol. They are present in non-filtered coffee, including boiled coffee, and Turkish coffee, but are largely removed by filtering the coffee (Urgert 1997). However, the phenolic compounds may exert cardio protective activities. The current habit of drinking filtered coffee, where the diterpenes are absent, may result in more beneficial effects
compared to the consumption of boiled, unfiltered coffee. In order to investigate the coffee effects on cardiovascular health, several studies has been performed. More recent meta-analysis have founded a U-shaped correlation among cardiovascular risk and coffee intake, with the lowest risk at 3–5 cups per day (Freedman 2012). The coffee consumption by healthy subjects has been observed resulted in increased expression of SR-BI and ABCG1 in macrophages, increasing 1.4 fold increase of cholesterol efflux. Concomitantly, sera collected after the intake of coffee was enriched with phenolic compounds, and promoted cell cholesterol efflux in cultured THP-1 macrophages more efficiently than pre-coffee sera. In vitro and in vivo investigations revealed that incubation of macrophages with ferulic or caffeic acid increased the cholesterol efflux to HDL, whereas the administration of ferulic acid to mice caused the elimination of cholesterol via the RCT pathway (Uto-kondo 2010).

Another important beverage is the tea. It is a very popular beverage worldwide, existing in six types (white, yellow, green, oolong, black, and post-fermented), that have different polyphenolic qualitative/quantitative compositions. Particularly oxidised black tea contains theaflavins and thearubigins, whereas non-oxidised green tea is a source of catechins, especially epigallocatechin-gallate. The beneficial impact of tea consumption is controversial, especially for the HDL cholesterol. Indeed it has been observed both increased and unchanged levels have been observed in subjects fed green tea (Zanotti 2015). Several mechanisms of beneficial effects of tea have been investigated in different animal models. An increase in faecal lipid elimination was observed and a decrease in cholesterol synthesis and LDLR activity and expression (Sae-tan 2011). The most active compound in tea is epigallocatechin-gallate, which is able to increase cholesterol faecal elimination in vivo increasing hepatic ABCG5/G8 expression (Hirsova 2012). In addition, some reports have suggested that even black tea consumption may have an atheroprotective effects in humans by reducing plasma lipid levels. This could be related to the ability of theaflavins and their metabolites to reduce lipid uptake from the liver and to decrease the cholesterol solubility in micelles in vitro and in vivo (Lin 2007).
PART I:

Evaluation of hypolipidemic activity of Bifidobacterium bifidum PRL2010 in vitro and in vivo
In recent years, new ways to treat diseases is spreading, aimed to support the medical therapy or replace it. One of the major public health problems in the western world is the patients’ compliance to medical treatment. Indeed World Health Organization (WHO) has estimated that 38% of patients on short term treatment and 43% of patients on long term treatment do not adhere until the end of medical therapy (Ngoh 2009). Several causes induce a reduced adherence to the therapy, as the complexity of treatments and the side effects of administered drugs, especially for chronic diseases. In order to avoid the abandonments of therapies, it may be used a nutraceutical approach supporting pharmacological drugs or in some cases, replace them. The co-administration of nutraceutical compounds may imply the reduction of co-administered doses of drugs, and consequently a decrease of adverse effects of pharmacological drugs. Especially for the chronic therapies, the reduction of side effects induces a reduced number of abandonments of drugs treatments, and an improvement of living condition of patients.

Nutraceutical therapies include molecular complexes and microorganisms, which come from plants, milk, and functional foods. The administration of adequate amounts of microorganism, as probiotic bacteria, are recently used in several diseases as gastrointestinal disease, diarrhoea, Clostridium difficile infections (Sharma 2014). In addition to improving gut health, probiotics have also been documented to exert other effects as the support of the immune system (Frei 2015), anti-oxidative effects (Songisepp 2004), reduction of allergic symptoms (Di Gioia 2014) and improvement of vaginal candidiasis in women (Falagas 2006). In addition, it has been discovered that some strains have cholesterol-lowering effects on lipid profile of the host. Indeed probiotics may modulate the lipid profile of people presenting high plasmatic levels of cholesterol, which are associated with cardiovascular diseases (CVD), that are the major cause of death in western world (Santos-Gallego 2011). Until now, several Lactobacilli and Bifidobacteria strains are used as probiotics because are most common strains of human gut. As literature suggested, there are several studies which have demonstrated the cholesterol lowering effects of Lactobacilli and Bifidobacteria strains, in animal models and in human clinical trials. Experiments conducted in pigs by Gilliland et al. have demonstrated the cholesterol lowering capacity of L. acidophilus P-47 and RP-32.
An important clinical trial demonstrated the activity in hypercholesterolaemic subjects of a yogurt formulation containing *Lactobacillus reuteri* NCIMB 30242, which reached significant reductions in LDL-cholesterol, total cholesterol, non-HDL-cholesterol, and a significant change in apoB (Jones 2012).

Other researchers focused on the activities of bifidobacteria strains. The cholesterol lowering effects of several strains belonging bifidobacteria genera have been observed in rats. The administration of the probiotic mixture of *B. bifidum* MB 109, *B. breve* MB 113, and *B. animalis* subsp. *lactis* MB 2409 has shown a significant reduction of total cholesterol and LDL, mostly due to cholesterol assimilation in bacterial cells, and Bile Salt Hydrolases (BSH) activity. Former mechanism reduces the amount of cholesterol uptaken by enterocytes, latter mechanism hydrolyses the bile salt, preventing enterohepatic circulation (Bordoni 2013). Effects on lipid profile by bifidobacteria has been observed in human as well. Guardamanga et al. founded a slight reduction of total cholesterol and LDL in 38 dyslipidemic children, treated with a mix composed by three bifidobacteria (*B. animalis lactis* MB2409, *B. bifidum* MB109B, and *B. longum* longum BL04) (Guardamagna 2014). Other interesting example of bacterial activity on lipid profile in human is the meta-analysis of thirteen clinical trials. The administrations of *L. bulgaricus*, *L. sporogenes*, *E. faecium*, *B. lactis* and *B. longum* have significantly lowered LDL-C, total cholesterol, and triglycerides (Guo 2011).

The aim of our research is the study of bifidobacteria capabilities to reduce cholesterol levels in vitro and in vivo. In particular, we focused on *Bifidobacterium bifidum* PRL2010 (LMG S-28692), in order to investigate the bacterial mechanism of cholesterol lowering, the fate of cholesterol in the bacterial cells and the kinetic of the uptake process. PRL2010 was selected and provided by research team of prof. Marco Ventura, of Probiogenomic laboratory of University of Parma. First, we examined through in vitro experiments, the capability of *Bifidobacterium bifidum* PRL2010 to reduce cholesterol levels in synthetic growth media. In addition, with the intention to examine the cholesterol uptake mechanisms of this bacteria, we analysed the activities of genes of transporters involved in cholesterol uptake as ATP Binding Cassettes transporters. In terms of study the cholesterol metabolism of
bacteria, it has been examined the activity of reductase enzymes, which convert cholesterol to coprostanol (Lye 2010). Furthermore the fatty acids, which are crucial for the fluidity of membrane, were analysed. The alteration of the fatty acid profile is due to the cholesterol assimilation, which increase the ratio between total saturated and unsaturated acids (Kimoto 2002).

We analysed the activity of the Bile Salt Hydrolase, important enzyme converting bile acids in their deconjugated counterparts, which are more efficiently excreted into the feces. This bacterial process has been associated with the reduction of circulating cholesterol levels in humans (Ruiz 2013).

So as to investigate the effects of *Bifidobacterium bifidum* PRL2010 in animals, in vivo experiments were carried out. First part of animal experimentation has conducted with a wild type animal model, the C57BL/6 mouse. The schedule of treatment was the daily administration of *Bb* PRL2010, via gavage for four weeks.

In the second part of experiments, the effect on lipid profile of PRL2010 has been investigated in an atherosclerosis animal model, the ApoE knockout mouse. Deletion of Apolipoprotein E, involved in cholesterol homeostasis, leads to spontaneous hypercholesterolemia in mice (Zhang 1992).

After animals’ sacrifice, cholesterol efflux capacity of sera has been analysed, in order to examine the capacity of murine plasma to stimulate the process involved in the first step of reverse cholesterol transport (RCT).
Materials and Methods
5.2.1 Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in table 1. Bacteria were selected and provided by research team of prof. Marco Ventura, of Probiogenomic laboratory of University of Parma.

Bifidobacterial cultures were incubated in an anaerobic atmosphere [2.99% (v/v)H₂, 17.01%(v/v) CO₂, and 80%(v/v)N₂] in a chamber (Concept 400, Ruskin) in deMan-Rogosa-Sharp (MRS) (Scharlau Chemie, Germany) supplemented with 0.05% (w/v) L-cysteine hydrochloride and incubated at 37 °C for 16 h.

<table>
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<th>Strains</th>
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<tr>
<td><em>B. bifidum</em> PRL2010 (LMG S-28692)</td>
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<tr>
<td><em>B. bifidum</em> LMG11041</td>
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<td><em>B. bifidum</em> 85B</td>
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<td><em>B. breve</em> 12L</td>
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<td><em>B. breve</em> LMG13208</td>
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<td><em>B. adolescentis</em> 22L</td>
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<td><em>B. adolescentis</em> ATCC15703</td>
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<tr>
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<tr>
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<td><em>B. pseudocatenulatum</em> LMG10505</td>
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Table 1: Bifidobacteria strains used during the screening

5.2.2 Bacterial Cholesterol Uptake

Cholesterol uptake by different bifidobacterial strains was assessed by a radioisotopic method, using ^3^H-cholesterol (Perkin Elmer, Italy) as a tracer.
Bifidobacteria were grown in MRS medium until they have reached exponential phase (optical density: 0.6). At this stage $^{3}$H-cholesterol was added to the culture medium at final concentration of 2μCi/ml together with micelles constituted by cholesterol 100 μg/ml and sodium taurocholate 5 mM or 0.25% of Ox gall, which is greenish-brown liquid mixture containing cholesterol, lecithin, taurocholic acid, and glycocholic acid (Sigma-Aldrich, U.S.A). In addition, thermally inactivated cells were obtained following exposure of exponential phase (OD 0.6) PRL2010 cells to 90 °C for 10 min. Loss of viability was checked by plating an aliquot of thermally inactivated cells on MRS agar.

Samples were collected after 3 hours and monitored for putative incorporation into bacterial cells. Cultures were centrifuged at 2,000g for 15min and pellets were lysed with 30 mg/ml of lysozyme and 1 mg/ml of mutanolysin for 3 h at 37 °C. The degraded cell wall fraction contained in supernatant was collected after centrifugation at 10,400g for 10min of the suspension. The pellet, representing the peptidoglycan hydrolysate, as well as membrane and cytosolic fraction, was suspended in water and added by 1:10 (v/v) 10% SDS. The radioactivity incorporated into the peptidoglycan hydrolyzed and membrane-cytosolic fractions were measured by liquid scintillation counting.

### 5.2.3 Quantification of Cholesterol, Coprostanol, and Fatty Acids

The relative quantitative analysis of free fatty acids, cholesterol, and coprostanol was performed by liquid chromatography-mass spectrometry (LC-MS). The chromatographic separation was performed on a Sinergy Fusion C18 (50×2.1 mm, 4 um) column (Phenomenex, CA, USA) by employing an HP1200 Agilent LC system (Agilent Technologies, USA) equipped with an electrospray QTRAP 4,000 mass spectrometer (ABSCIEX). Mobile phase were 0.05 mol/l ammonium acetate and acetonitrile, and elution was under isocratic conditions (30–70 %, A-B). The mobile phase was filtered through a 0.45-μm cellulose membrane before use and delivered at a flow rate of 0.2 ml/min. Injected volume was 5μl. The system was controlled by the Analyst software v1.4.

Source parameters were as follows: ESI voltage, 4.5 kV; declustering potential, 50 V; entrance potential, 10 V; and source temperature, 400C. Quadrupoles were tuned
Part I – Materials and Methods

5.2.4 Bile Salt Hydrolase Activity Assays

Bile salt hydrolase (BSH) activity was qualitatively determined on glycocolcholate (GC), glycodeoxycholate (GDC), taurocholate (TC), and taurodeoxycholate (TDC) (Sigma-Aldrich, U.S.A.). A sample of 0.5M of the corresponding bile salt was added to MRS agar plates supplemented with 0.25 % L- cysteine (w/v), and the hydrolyzing activity was tested according a previously published procedure (Noriega 2006). Briefly, overnight cultures were washed two times with phosphate-buffered saline (PBS) and adjusted to an Abs 600nm of 1.0 in Phosphate Buffer Solution (PBS). Ten microliters of cell suspensions was placed on the top of the plates and incubated for 48 h at 37 °C in anaerobic conditions. Production of opaque white colonies due to bile salt precipitation was considered to be a positive result for the assessment of this activity. BSH activity was quantified on the salts showing a positive result in the qualitative test. The determination of the BSH activity was obtained by quantifying the amount of glycine or taurine released from the conjugated bile salts. Conjugated bile salts were incubated in a BSH reaction mix with the cell-free extract, in order to achieve the release of the amino acids, according to an established method (Tanaka 1999). Five microliters from the BSH reaction mix (or 5μl of the glycine/taurine standards or blanks, depending on the assay) was diluted five times with 0.5M sodium citrate buffer, pH 5.5, and mixed with 110μl of ninhydrin reagent. Reactions were incubated for 14 min at 97°C and cooled down immediately to 4 °C. Absorbance at 570 nm was measured in an Epoch™ Spectrophotometer (Biotek, Italy) and standard curves of glycine/taurine were calculated. One unit of BSH activity was defined as the amount of protein able to release 1μmol of glycine/taurine per minute. The amount of protein present in the different extracts was measured.
with the BCA Protein Assay kit (Thermo Fisher Scientific, U.S.A.). Experiments were performed in three independent trials and data expressed as BSH-specific activity (units/mg protein).

5.2.5 Murine colonization
All animals used in this study were housed in animal facility of University of Parma, in compliance with guidelines established by the Italian Ministry of Health. All procedures were approved by the University of Parma, as executed by the Institutional Animal Care and Use Committee (Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti Direzione Generale della Sanità Animale e del Farmaco Veterinario).

Mice were housed in a controlled area with constant temperature of 21°C, humidity of 45±5%, and a cycle of 12 hours light/dark and were fed ad libitum with a standard chow diet (Mucedola, Italy). Two groups of 10-weeks-old male ApoE knockout and C57BL/6 mice (Charles River Laboratories, Italy) were orally inoculated with bacteria (treated group; n=5) or water (control group; n=5) for 20 days. Bacterial colonization was established by consecutive daily administrations whereby each animal received a dose of $10^9$ cells of overnight cultures of PRL2010 by oral gavage.

As shown in graph 1, murine faecal samples were collected daily during in vivo trial and subjected to DNA extraction using the QIAampDNA Stool Mini kit following the manufacturer’s instructions (Qiagen, Germany). Blood was collected by tail puncture and, after the sacrifice, by cardiac puncture, and recovered in plastic tubes containing heparin. Plasma was isolated by low-speed centrifugation and stored at −80 °C until use. The intestinal colonization of B. bifidum PRL2010 was measured on faecal samples following the protocol previously described (Turroni 2013).

**Graph 1**: Time table of faeces, blood collections and monitoring of weight
5.2.6 Evaluation of Plasma Cholesterol and Triglycerides In Mice

Blood samples were collected from tail vein at baseline and by cardiac puncture at sacrifice time. The blood was then centrifuged at 5,000g for 15 min at 4°C to isolate plasma, and then stored at -80°C. Total cholesterol, HDL cholesterol, and triglycerides in plasma were quantified using commercially available kits (IL TEST™, Instrumentation Laboratory, Italy) according to the manufacturer’s instructions.

5.2.7 Evaluation of Plasma Efflux Capacity

Plasma was slowly defrosted in ice just before addition to cells as extracellular acceptors in cholesterol efflux experiments.

5.2.7.1 Measurement of Passive Diffusion And ABCA1-mediated Cholesterol Efflux

Efflux by passive diffusion and ABCA1 mediated efflux was measured using J774 macrophage treated with cpt-cAMP to up-regulate ABCA1 (Bortnick 2000). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Switzerland) with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, U.S.A), incubated at 37°C in 5% CO₂, seeded in 12-well plates and utilized at 80-90% confluence. Monolayers were radiolabeled with [³H]-cholesterol 2µCi/mL (Perkin Elmer, Italy) in medium containing 1% FCS. Following 24 hours labelling period, cells were washed and incubated with 0.2% of Bovine Serum Albumins (BSA, Sigma-Aldrich, U.S.A), with or without 0.3mM cpt-cAMP (Sigma-Aldrich, U.S.A.) for 18 hours. 2 µg/ml of ACAT inhibitor (Sandoz 58035, Sigma-Aldrich, U.S.A.) was added during labelling and equilibration period to prevent cellular accumulation of cholesteryl ester (Zanotti 2012). After equilibration period cells were incubated with 1% (v/v) plasma isolated from mice for 4 hours. The radioactivity in the medium was determined by liquid scintillation counting. Cholesterol efflux was calculated as a percentage of the radioactivity released into the medium over the radioactivity incorporated by cells before addition of plasma (Time zero). In order to analyse cellular [³H]-cholesterol content, cell monolayers were extracted by the addition of 0.6 ml of 2-propanol
(VWR International, U.S.A.). The lipid extracts were dried under a stream of N₂, re-suspended in toluene (VWR International, U.S.A.), and quantified by liquid scintillation counting. Every plasma sample was analysed in triplicate and the average and standard deviation have been obtained. The ABCA1-mediated cholesterol efflux was calculated as the percentage efflux from stimulated J774 macrophages minus the percentage efflux from not stimulated J774 cells.

5.2.7.2 Measurement of ABCG1-mediated Cholesterol Efflux

Chinese Hamster Ovary cells (CHO-K1) cells expressing human ABCG1 were used to measure ABCG1-mediated cholesterol efflux and were generated as previously described (Gelissen 2006). The ABCG1-mediated cholesterol efflux was calculated as the percentage efflux from transfected cells minus the percentage efflux from CHO-K1 cells, which do not express the vector containing ABCG1 gene. Both cells lines were seeded in 24 well-plates and labelled for 24h with [³H]-cholesterol 2µCi/mL in medium containing 10% FCS. Following 24h cells were washed, and equilibrated for 90 minutes in serum-free medium, then incubated in efflux medium containing BSA (1mg/ml) in the presence of 1% (v/v) plasma isolated from WT and ApoE knockout mice for 6h. Cells and media were assayed for radioactivity. Cholesterol efflux was calculated as a percentage of the radioactivity released to the medium in 6h over the radioactivity incorporated by cells before addition of plasma (Time zero). To analyse cellular [³H]-cholesterol content, cell monolayers were extracted by the addition of 0.6 ml of 2-propanol. The lipid extracts were dried under a stream of N₂, re-suspended in toluene, and quantified by liquid scintillation counting. Every plasma sample was analysed in triplicate and the average and standard deviation have been obtained.

5.2.7.3 Measurement of SR-BI-Mediated Cholesterol Efflux

SR-BI-mediated efflux was tested in rat hepatoma Fu5AH cells which highly express SR-BI transporter (de la Llera-Moya 1999). Cells were seeded in 24-well plates and radiolabeled with [³H]-cholesterol 2µCi/mL in DMEM medium containing 1% FCS
for 24h. Cells were then equilibrated in BSA 0.2% containing medium. During labelling period and equilibration period cells were exposed to 2 µg/ml of ACAT inhibitor (Sandoz 58035, Sigma-Aldrich, U.S.A) to ensure that all labelled cholesterol was present as free cholesterol. Cells were then washed and incubated with 2.5% (v/v) plasma from WT and ApoE knockout mice for 4h. The radioactivity in the medium was determined by liquid scintillation counting. Cholesterol efflux was calculated as a percentage of the radioactivity released to the medium in 4h over the radioactivity incorporated by cells before addition of plasma (Time zero). Cell monolayers of baseline (time 0) were extracted by the addition of 0.6 ml of 2-propanol. The lipid extracts were dried under a stream of N2, re-suspended in toluene, and quantified by liquid scintillation counting. Every plasma sample was analysed in triplicate and the average and standard deviation have been obtained

5.2.8 RNA Isolation and Transcriptomic Analysis

The experiments below have been performed in the Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parma, Italy. Two conditions were analyzed, the former involving B. bifidum PRL2010 cells that were grown to an OD of 0.6, followed by the addition of cholesterol, the latter including 5% oxgall and 100 µg/ml cholesterol and subsequent incubation for 180 min, after which RNA was harvested and labeled for microarray analysis. Total RNA was isolated using the methods described previously (Turroni 2010). Briefly, cell pellets were re-suspended in 1 ml of QUIAZOL (Qiagen, United Kingdom) and placed in a tube containing 0.8 g of glass beads (diameter: 106 µm; Sigma-Aldrich, U.S.A.). The cells in this suspension were disrupted by shaking the mix on a BioSpec homogenizer at 4 °C for 2 min. The resulting mixture was then centrifuged at 12,000 rpm for 15 min, and the upper phase containing the RNA-containing sample was recovered. The RNA sample was further purified by phenol extraction and ethanol precipitation according to an established method (Sambrook 2012). Quality and integrity of the RNA was checked by Experion (Bio-Rad) analysis. RNA concentration and purity were then determined with a Bio-Rad Smart Spec Spectrophotometer, and the quality and integrity of the RNA were checked by Experion (Bio-Rad) analysis.
5.2.9 Microarray, Description, Labeling, Hybridizations, and Data Acquisition

The experiments Below have been performed in the Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parma, Italy.

Microarray analysis was performed with an oligonucleotide array based on the *B. bifidum* PRL2010 genome: a total of 39,249 oligonucleotide probes of 35 bp in length were designed on 1,644 ORFs using Oligo Array 2.1 software (Rouillard 2003). The Oligos were synthesized in triplicate on a 2×40 k CombiMatrix array (CombiMatrix, U.S.A.). Replicates were distributed on the chip at random, non-adjacent positions. A set of 74 negative control probes designed on phage and plant sequences were also included on the chip. Reverse transcription and amplification of 500ng of total RNA were performed with MessageAmp II-Bacteria kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Five micrograms of RNA was then labeled with ULS Labelling kit for Combimatrix arrays with Cy5 (Kreatech, Netherlands). Hybridization of labeled DNA to *B. bifidum* PRL2010 arrays was performed according to CombiMatrix protocols (http://www.combimatrix.com/support_docs.htm).

Fluorescence scanning was performed on an InnoScan 710microarray scanner (Innopsys, France). Signal intensities for each spot were determined using GenePix Pro 7 software (Molecular Devices, USA). Signal background was calculated as the mean of negative controls plus two times the standard deviation (Bilban 2002).

5.2.10 Statistical Analysis

Values are expressed as mean ± SD. Statistical analyses were performed with Prism 5 software (GraphPad Software, San Diego, California). Statistically significant differences among the means of different groups were tested using analysis of variance (ANOVA one way). The Bonferroni test was performed after ANOVA and p value of <0.05 was considered significant.
Results
5.3.1 Cholesterol Intake by Actively Growing Bifidobacterial Cultures

As mentioned in literature, several studies have demonstrated the ability of small number of bifidobacterial strains as *B. bifidum*, *B. breve*, and *B. animalis* subsp. *Lactis* to assimilate cholesterol (Grill 2000; Pereira 2002; Bordoni 2013). Cholesterol intake from probiotics, reduces the amount of cholesterol captured by enterocytes, and therefore may reduce plasma levels of cholesterol in the host. For this reason we decided to analyse cholesterol assimilation of various bifidobacterial strains by monitoring the fate of $^3$H-labeled cholesterol when supplemented to bacterial cultures.

5.3.1.1 Standardization of an in Vitro Method for Evaluation of Cholesterol Intake

First step was the standardization of an in vitro method for the evaluation of cholesterol uptake from the bacteria, which must be the condition more physiological similar to the intestinal lumen of humans. Therefore we analysed the cholesterol assimilation ability of a bifidobacteria strain as *Bifidobacterium bifidum* PRL2010, in MRS in three different conditions of cholesterol administration:

- cholesterol 250 μM + EtOH anhydrous, which is the common solvent of cholesterol powder; despite this is the less physiological condition;
- cholesterol 250 μM + Oxgall 0,25%, which is a dehydrated fresh bile and is used specifically for differentiation of bile tolerant microorganisms (Murray 1995), in addition it partially mimics the intestinal environment (Parvez et al. 2006);
- cholesterol 250 μM + Sodium Taurocholate Hydrate 5 mM, which is the more physiological condition that reproduces how cholesterol is present in the intestinal lumen, namely in form of micelles, that are captured by enterocytes (Ikeda 2015).

Cholesterol solutions and $[^3]$H-cholesterol were added to the culture at early exponential phase. Cells were harvested after 3 hours and monitored for putative incorporation into living cells. As shown in graph 2, a significant increase in
Radioactivity was noticed for micelle condition, compared with Ox gall condition and with ethanol condition.

In order to understand if the cholesterol uptake is due to a passive diffusion or transporters mediated, we compared the cholesterol intake of thermally inactivated Bifidobacterium bifidum PRL2010 (exposing them to 80°C for 30 minutes) with the alive bacterium, administering the three different cholesterol solution described previously. As the graph 3 indicates, the cholesterol uptake decreased in thermally inactivated bacteria compared to live bacteria both in ox-gall condition than in taurocholate condition. Conversely, the cholesterol uptake of thermally inactivated bacteria resulted increased, after administration of ethanol solution of cholesterol.

We can state that the captured cholesterol in bacteria cells is mainly due to active...
transport and not by passive diffusion when cholesterol is administered in “physiological” condition, as ox-gall and micelle composed by taurocholate.

Graph 3: The cholesterol uptake of Bifidobacterium bifidum PRL 2010 in living conditions vs thermic inactivation with three different conditions of cholesterol administration: cholesterol 250 μM + EtOH anhydrous, Oxgall 0.25% or Sodium Taurocholate Hydrate 5 mM, incubated with $^3$H-cholesterol for 3 hours. Cholesterol intake values were expressed as percentage of uptake/cells x 10^8 ± SD. Significance: *** = p< 0.001.

5.3.1.2 Screening of Bacterial Strains Aimed to Identify Strains with a Significant Cholesterol Uptake Ability

We made a screening of bifidobacteria and lactobacilli strains which mostly form the human microbiota, aimed to identify the most active bacteria for cholesterol uptake. We used the standardized method described above. Strains were grown in MRS, and 2μCi/mL of $[^3]$H-cholesterol, and cholesterol-taurocholate micelles, were added to the culture at early exponential phase. Samples were collected after 3 hours and monitored for putative incorporation into living cells. As shown in graph 4, a significant increase in radioactivity was noticed for the B. bifidum species. This suggests that these bacteria possesses a higher cholesterol assimilation ability.
compared with the examined bifidobacterial taxa as *B. animalis* subsp. *lactis* or *B. longum* subsp. *infantis* (p value <0.01).

**Graph 4:** Accumulation of tritiated cholesterol by living cells belonging to different bifidobacterial species. The y-axis represents the normalized uptake (%) of radiolabeled cholesterol. The uptake was calculated based on the radioactivity incorporated by each strain and normalized to the radioactivity incorporated by *B. bifidum* PRL2010 as expressed in percentage, where the latter was set at 100 %. Significant differences in expression levels are indicated with an asterisk (p≤0.01)

### 5.3.1.3 Evaluation of Cholesterol-Lowering Activities of *Bifidobacterium bifidum* PRL2010 as Representative of *B. bifidum* Species

In order to better exploit the molecular features sustaining this interesting phenotype, we decided to focus on *B. bifidum* PRL2010, which represents our prototypical human gut bifidobacterial strain (Serafini 2014; Turroni 2009). Indeed, we evaluated the radiolabeled cholesterol incorporation of *Bb* PRL2010 cells in 28 hours of exposition with micelles composed by $^3$H-cholesterol and taurocholate, comparing thermally inactivated bacteria versus alive condition.
Under the conditions applied, *B. bifidum* PRL2010 enters into the stationary phase after 24 hours, whereas thermal inactivation resulted in cell death (graph 5). As graph 6 showed, living bacterial cells have shown a significant and rapid increase of $^3$H-cholesterol assimilation, compared with inactivated *Bb* PRL2010. In addition, we detected a weak cholesterol amount into the thermally inactivated bacterial cells, probably due to a passive cholesterol process, rather than an active (transporters mediated) uptake mechanism (graph 6) (Kimoto 2015). In order to identify the fate of the assimilated cholesterol in *Bb* PRL2010 cultures, the radiolabelled cholesterol amount present in peptidoglycan layer after enzymatic hydrolysis, has been compared with the remaining cytosolic fraction. Results have shown that ≥98 % of the cholesterol-associated radioactivity was found into the cellular cytosol, suggesting that a large part of cholesterol was incorporated into the bifidobacterial cells (graph 7).

**Graph 5:** Viability of *B. bifidum* PRL2010 cells over a time period of 30 h using both living and thermally inactivated cells (exposing them to 80°C for 30 minutes). Living cell condition display a rapid increase of cell number, reaching the plateau after 18 hours of incubation with cholesterol, compared with thermally inactivated ones. Data are expressed in bacterial cells per ml.
5.3.2 Effect of Cholesterol Uptake on Membrane Fatty Acid Composition

Cholesterol exerts a crucial role in biological membranes; indeed it is involved in the modulation of membrane fluidity by interacting with phospholipids. It is well known that cholesterol and fatty acids exert a crucial role in biological membranes through their ability fluidity modulation which occurs in response to environmental stimuli.
Cholesterol assimilation causes an alteration of the fatty acid profiles increasing the ratio between total saturated and unsaturated acids (Kimoto 2002).

In order to understand if the cholesterol uptake ability of alive *B. bifidum* PRL2010 cells is related to the incorporation of cholesterol into the cell membrane and a consequent alteration of its composition, isolated membrane lipid profiles were assayed after cells exposure of 3 hours to supplementation of micelle containing cholesterol 100 μg/ml and sodium taurocholate 5 mM, and compared to those of *B. bifidum* PRL2010 cells grown in the absence of cholesterol. As graphs 8 and 9 show, the saturated stearic acid (C18:0), the mono-unsaturated palmitoleic (C16:1) and oleic acids (C18:1), the polyunsaturated linoleic (C18:2) and eicosadienoic acids (C20:2) were increased after cell exposure to cholesterol-sodium taurocholate micelles (graph 7-8). Our findings suggest that *Bb* PRL2010 exposure to high concentrations of cholesterol results in the modification of the physical features of the cell membrane such as fluidity and its associated permeability.

Graph 8: Distribution of saturated fatty acids in the bacterial membrane after cell exposure of 3 hours to two conditions: the presence or absence of cholesterol-sodium taurocholate micelles. Saturated fatty acids values were expressed as mass/cells ± SD. Significance: * = p< 0.05.
5.3.3 Cholesterol Degradation after Bacterial Uptake

Graph 10 described the amount of coprostanol detected in cells grown in the presence of micelle enriched in cholesterol was significantly higher compared to untreated samples. This data may be interesting, because coprostanol is described to represent a key derivative of cholesterol degradation, whose formation is associated with reduction of plasma cholesterol levels in humans (Gerard 2007).
5.3.4 *B. bifidum* PRL2010 Bile Salt Hydrolase Activity

As mentioned previously, bile salt hydrolases (BSH) is wide diffuse in bacteria composing intestinal flora. BSH activity induces the conversion of bile acids in their deconjugated counterparts, which are more efficiently excreted into the feces. This process has been associated with the reduction of circulating cholesterol levels in humans (Ruiz 2013). Thus, analysis of BSH activity in bifidobacteria may provide evidence for their potential hypocholesterolemic activity.

BSH activity of *B. bifidum* PRL2010 is measured as the deconjugation of known concentration (0,5 M) of various secondary bile salts as glycocholate (GC), glycodeoxycholate (GDC), taurocholate (TC), or taurodeoxycholate (TDC). As displayed in figure 10, Results have shown, the significant BSH activity on GDC (2.096 ± 0.880 units/m protein), and on TDC (7.075 ± 0.861 units/m protein).

These values seems to be similar to that observed in other Bifidobacterium species, such as *B. animal* and *B. longum* (Noriega 2006). In bifidobacteria, a high BSH activity has been correlated with the acquisition of bile resistance. Indeed amphiphilic nature of bile salts induces an inhibitory activity for bacteria proliferation and strongly influences bacterial survival in the gastrointestinal tract. Since the BSH activity is bacterial adaptation to bile stress of intestinal environment (Sanchez 2007). Remarkably, there is evidence of a link between bile salt hydrolysis and cholesterol lowering activity in probiotic bacteria, and some bacterial BSH producers have been promoted as potential cholesterol-lowering agents (Ishimwe 2014).
5.3.5 Transcriptome of *B. bifidum* PRL2010 cultures growing in cholesterol

The experiments below have been performed in the Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parma, Italy.

In order to investigate if the cholesterol treatment influence the genes expression of *B. bifidum* PRL2010, transcriptional profiling experiments were carried out. We first investigated the transcriptomes of *B. bifidum* PRL2010 cultures grown in MRS, compared with the transcriptomes of the bacteria incubated with cholesterol in the presence or absence of ox-gall.

As shown in graphs 11, the most significant transcription profiling results are those corresponding to the condition of *B. bifidum* PRL2010 incubated with ox-gall in the presence of cholesterol, compared with the condition without cholesterol. However, the transcriptome of latter condition overlaps with transcriptome achieved after the addition of cholesterol, whereas the only differences observed appeared to be due to ox-gall-induced genes.

In order to study in depth the effects of cholesterol incubation on the activity of bacterial genes, it has been decided to focus on the analysis of the bacterial cells...
cultivated in the presence of only cholesterol, which indicated that 47 genes exhibited at least a 2-fold increase in expression compared with the *B. bifidum* PRL2010 cultivated in absence of cholesterol, which represents the reference condition (Table 2).
Graph 11: transcriptome analysis of genes of *B. bifidum* PRL2010 which shown the differentially expressed genes by in response to the presence of cholesterol. Panel a represents a heat map displaying the change in *B. bifidum* PRL2010 gene expression in the presence of cholesterol or cholesterol plus oxgall or MRS. Each row represents a separate transcript and each column represents a separate sample. Green indicates increased transcription levels as compared to the reference samples. Dendrogram on the left margin of the heat map represents the Hierarchical Clustering algorithm result based on Average Linkage (UPGMA) and Euclidean distance of the gene dataset. Panel b is a Venn diagram showing the number of genes expressed during the different conditions indicated at the bottom of each circle. Panel c depicts functional annotation of the cholesterol-expressed genes of *B. bifidum* PRL2010 according to their COG categories.
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<td>BBPR_1111</td>
<td>Hypothetical protein</td>
<td>4.137</td>
<td>6.23E-04</td>
</tr>
<tr>
<td>BBPR_1197</td>
<td>Aminotransferase</td>
<td>2.170</td>
<td>5.87E-04</td>
</tr>
<tr>
<td>BBPR_1278</td>
<td>tRNA 2-methylthioadenosine synthase</td>
<td>2.365</td>
<td>7.78E-02</td>
</tr>
<tr>
<td>BBPR_1306</td>
<td>Conserved hypothetical protein</td>
<td>4.142</td>
<td>1.26E-03</td>
</tr>
<tr>
<td>BBPR_1348</td>
<td>Solute-binding protein of ABC transporter system for peptides</td>
<td>2.320</td>
<td>7.42E-04</td>
</tr>
<tr>
<td>BBPR_1410</td>
<td>Conserved hypothetical protein</td>
<td>3.069</td>
<td>5.87E-04</td>
</tr>
<tr>
<td>BBPR_1441</td>
<td>Narrowly conserved hypothetical membrane spanning protein</td>
<td>2.874</td>
<td>8.90E-03</td>
</tr>
<tr>
<td>BBPR_1508</td>
<td>PtsG PTS system. glucose-specific IIABC component</td>
<td>2.533</td>
<td>1.00E-03</td>
</tr>
<tr>
<td>BBPR_1599</td>
<td>Conserved hypothetical protein</td>
<td>3.627</td>
<td>5.87E-04</td>
</tr>
<tr>
<td>BBPR_1615</td>
<td>Pep Pyrrolidone-carboxylate peptidase</td>
<td>3.561</td>
<td>1.36E-03</td>
</tr>
<tr>
<td>BBPR_1662</td>
<td>GinR Transcriptional regulatory protein</td>
<td>2.260</td>
<td>6.41E-03</td>
</tr>
<tr>
<td>BBPR_1704</td>
<td>ATP-binding and permease protein of ABC transporter</td>
<td>2.795</td>
<td>3.38E-03</td>
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<tr>
<td>BBPR_1707</td>
<td>Fimbrial subunit FimA</td>
<td>2.690</td>
<td>7.42E-04</td>
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<tr>
<td>BBPR_1792</td>
<td>Haloacid dehalogenase-like hydrolase (HAD superfamily)</td>
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<td>6.99E-04</td>
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<td>hspR heat shock response regulator</td>
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<td>1.66E-02</td>
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<tr>
<td>BBPR_1836</td>
<td>Thioredoxin reductase</td>
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<td>2.473</td>
<td>8.63E-04</td>
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<tr>
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<td>LeuC 3-isopropylmalate dehydratase large subunit</td>
<td>2.042</td>
<td>8.40E-04</td>
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<tr>
<td>BBPR_0279</td>
<td>GltX Glutamyl-tRNA synthetase</td>
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<td>4.12E-03</td>
</tr>
<tr>
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<td>Ald/keto reductase family</td>
<td>2.310</td>
<td>6.23E-04</td>
</tr>
<tr>
<td>BBPR_0891</td>
<td>ParB Chromosome partitioning protein</td>
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<td>1.81E-03</td>
</tr>
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<td>Hypothetical protein</td>
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<td>6.06E-03</td>
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<td>2.984</td>
<td>5.87E-04</td>
</tr>
<tr>
<td>BBPR_1691</td>
<td>GinD [protein-PPI] uridylyltransferase</td>
<td>2.095</td>
<td>1.55E-02</td>
</tr>
</tbody>
</table>

Table 2: Genes exhibiting at least a 2.0-fold increase in expression following cholesterol incubation with respect to PRL2010 cultivated in absence of cholesterol
Five predicted transporter-encoding genes has been identified; in particular three are belonging to the ABC family (BBPR_0676, BBPR_1704, and the solute-binding protein BBPR_1348), one belongs to the MFS family (BBPR_0146), and one whose product is similar to EIIC component (BBPR_1508) of PTS system. In addition, the glycerol-3-phosphate dehydrogenase-encoding gene (specified by BBPR_0261) had a six-fold up-regulation in presence of cholesterol; this gene is involved in lipid biosynthesis and it may be implicated in the structural and functional integrity of membranes (Edgar 1978). Furthermore, the cholesterol-induced transcriptomes of *B. bifidum* PRL2010 cells included two genes (BBPR_0043 and BBPR_1441) encoding membrane-spanning proteins, which may be involved in membrane fluidity modulation upon cholesterol assimilation.

**5.3.6 In Vivo Analysis of Mice Treated with *B. Bifidum* PRL2010**

In order to assess if cholesterol uptake and metabolism observed in vitro by *B. bifidum* PRL2010 cells result in hypolipidemic activity under in vivo conditions, we tested *B. bifidum* PRL2010 in two different animal models, commonly used for atherosclerosis studies: C57BL/6 mouse, which is a non-transgenic animal model and apoE knockout mouse, which spontaneously develops atherosclerotic lesions on a standard chow diet, due to the lack of apolipoproteinE (Meir 2004).

The daily supplementation of 10⁹ cells of PRL2010 to apoE knockout and C57BL/6 mice for the entire time of treatment resulted in stable intestinal colonization (graphs 12a-b). At the end of the treatment period, in both animal models, no significant changes in body weights were observed among control and treated groups (graphs 13a-b).
Graph 12a: Intestinal colonization of *Bifidobacterium bifidum* PRL2010 in treated and in placebo group of C57BL/6 mice, during all days of the experimentation. Each point represents the average of n° bacterial DNA copies per grams of faeces.

Graph 12b: Intestinal colonization of *Bifidobacterium bifidum* PRL2010 in treated and in placebo group of ApoE knockout mice, during all days of the experimentation. Each point represents the average of n° bacterial DNA copies per grams of faeces.
5.3.6.1 Effects of Treatment with *B. Bifidum* PRL2010 on Lipid Profile of Mice

As described in the materials and methods chapter, the lipid profile of C57BL/6 and ApoE knockout mice has been analyzed at T0 and at the end of treatment. It has been quantified the total cholesterol, HDL and triglycerides values in placebo group and in the group receiving the *B. bifidum* PRL2010.

As described in the graph 13, the treatment does not influence significantly the lipid profile of C57BL/6 mice. Interestingly, the administration of PRL2010 to ApoE knockout mice appears to reduce total cholesterol in plasma \( p=0.059 \), without significantly affecting triglyceride or HDL levels (graph 14).
Graph 14: displays the average values of the total cholesterol, HDL, and triglycerides of the treated versus placebo group at T0 and after 20 days in C57BL/6 mice
Graph 15: displays the average values of the total cholesterol, HDL, and triglycerides of the treated versus placebo group at T0 and after 20 days in ApoE knockout mice.
5.3.6.2 Evaluation of Plasma Efflux Capacity of Mice Treated with *B. Bifidum* PRL2010

Cholesterol efflux from cells is the rate limiting step of macrophage RCT, as it regulates the amount of cholesterol released from foam cells of the arterial wall and available to be delivered to the liver for disposal (Rader 2009). In order to investigate if the administration of *B. bifidum* PRL2010 might influence cholesterol efflux from foam cells of atherosclerotic plaque, experiments have been performed using the plasma of C56BL/6 and ApoE knockout mice after sacrifice, on cells line that express the transporters ABCA1, ABCG1 and SR-BI, respectively J774, CHO-G1/K1 and FU5AH.

- *Cholesterol Efflux of Plasma of C57BL/6 Mice*

**Graph 16:** This graphic shows the efflux ABCA1 mediated of plasma of C57BL/6 mice treated with placebo or with *B. bifidum* PRL2010. J774 macrophage were radiolabeled with \[^3\text{H}\]-cholesterol for 24 hours, in DMEM medium with 1% FCS and 2µg/mL of an ACAT inhibitor. Then, cells were incubated for 18h with 0.2% BSA in presence of 0.3mM cpt-cAMP, washed, and incubated for 4h with 1% plasma isolated from C57BL/6 mice treated with placebo or with *Bifidobacterium bifidum* PRL2010. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0 x100± SD. Furthermore efflux values of passive diffusion and total efflux has been shown. Delta ABCA1 is obtained from difference among total efflux and passive diffusion.
Graph 17: This graphic shows the efflux ABCG1 mediated of plasma of C57BL/6 mice treated with placebo or with *B. bifidum* PRL2010. Wild-type and human ABCG1-overexpressing CHO cells were labeled with 1 μCi/mL [3H]-cholesterol in HAM’s medium with 10% FCS for 24 hours. Then, cells were washed, equilibrated for 90 min in serum-free medium, and incubated for 6 h with 1% plasma isolated from C57BL/6 mice treated with placebo or with *Bifidobacterium bifidum* PRL2010. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0 x 100 ± SD. The ABCG1-mediated cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells and the percentage efflux from CHO-K1 parent cells.

Graph 18: This graphic shows the efflux SR-BI mediated of plasma of C57BL/6 mice treated with placebo or with *B. bifidum* PRL2010. Cells were labeled with 2 μCi/ml [3H]-cholesterol for 24 h in DMEM medium with 1% FCS and 2 μg/ml of an ACAT inhibitor. Cells were then incubated for 18 h with 0.2% BSA, washed and incubated for 4 h with 1% plasma isolated from C57BL/6 mice treated with placebo or with *Bifidobacterium bifidum* PRL2010. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0 x 100 ± SD.
As graph 16 shown, ABCA1 efflux, total efflux, and passive diffusion mediate by sera of mice treated with *B. bifidum* PRL2010 is similar to the efflux values of placebo group. Graph 17 discloses that the ABCG1 efflux mediated of placebo group is higher compared with treated group. In addition, graph 18 shows that the SR-BI mediated efflux in treated group have analogous values of placebo group.

**Graph 19:** This graphic shows the efflux ABCA1 mediated of plasma of ApoE knockout mice treated with placebo or with *B. bifidum* PRL2010. J774 macrophage were radiolabeled with [³H]-cholesterol for 24 hours, in DMEM medium with 1% FCS and 2µg/mL of an ACAT inhibitor. Then, cells were incubated for 18h with 0.2% BSA in presence of 0.3mM cpt-cAMP, washed, and incubated for 4h with 1% plasma isolated from ApoE knockout mice treated with placebo or with *Bifidobacterium bifidum* PRL2010. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0 x100± SD. Furthermore efflux values of passive diffusion and total efflux has been shown. Delta ABCA1 is obtained from difference among total efflux and passive diffusion.
- **Cholesterol Efflux of Plasma of ApoE knockout mice**

**Graph 20:** This graphic shows the efflux ABCG1 mediated of plasma of ApoE knockout mice treated with placebo or with *B. bifidum* PRL2010. Wild-type and human ABCG1-overexpressing CHO cells were labeled with 1 μCi/mL [³H]-cholesterol in HAM’s medium with 10% FCS for 24 hours. Then, cells were washed, equilibrated for 90 min in serum-free medium, and incubated for 6 h with 1% plasma isolated from knockout mice treated with placebo or with *Bifidobacterium bifidum* PRL2010. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time ± SD. The ABCG1-mediated cholesterol efflux was then calculated as the difference between the percentage efflux from transfected cells and the percentage efflux from CHO-K1 parent cells.

**Graph 21:** This graphic shows the efflux SR-BI mediated of plasma of apoE knockout mice treated with placebo or with *B. bifidum* PRL2010. Cells were labeled with 2μCi/ml [³H]-cholesterol for 24h in DMEM medium with 1% FCS and 2μg/ml of an ACAT inhibitor. Cells were then incubated for 18h with 0.2% BSA, washed and incubated for 4h h with 1% plasma isolated from knockout mice treated with placebo or with *Bifidobacterium bifidum* PRL2010. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time ± SD.
As graph 19 shown, a slight increase of total efflux in the treated group is observed, whereas the ABCA1 efflux mediated of J774 cells treated with *B. bifidum* PRL2010 have comparable efflux values of placebo group.

Graph 20 discloses that the capacity of efflux of sera of placebo group have similar ABCG1 efflux mediated compared with treated group. Conversely the graph 21 shows an increase in SR-BI efflux mediated of mice treated with *Bifidobacterium bifidum* PRL2010, values obtained deducting from total efflux the values obtained with an antagonist of SR-BI, the BLT-1. In addition, the total efflux slight is increased in the treated group than in the control group.
Discussion
Cholesterol is an essential constituent of animal and bacterial cells, which modulates membrane fluidity and is the precursor of steroid hormones, vitamin D, and bile acids. Despite this, high levels in blood are widely recognized as a risk factor for cardiovascular disease (Goff 2006). Cardiovascular diseases (CVD) are one of the major causes of death in western world and atherosclerosis is the main risk factor for CVD. One of the major public health problems in the western world is the abandonment of therapies, mostly due to the adverse events or the complexity of treatments (Ngoh 2009). In order to avoid the desertion of pharmacological treatment, in recent years, nutraceutical approaches to treat CVD and in particular atherosclerosis is spreading, aimed to support the medical therapy or in case of low or medium hypercholesterolemia, replace it.

Nutraceutical therapies include the administration of molecular complexes or microorganisms, as probiotics. These bacteria are widely used in therapies against gastrointestinal disease, because they exert an immune-stimulating function, antioxidative effects and reduction of allergic symptoms (Frei 2015; Songisepp 2004; Di Gioia 2014). In addition, Shaper (1963) and later Mann (1974) discovered the cholesterol-lowering effects of several Bifidobacteria species, common strains of human intestine (Mann 1974). Indeed an increasing body of evidence has indicated that probiotic bacteria act as efficient hypocholesterolemic agents in humans (Jones 2012; Liong 2006; Pereira 2002). Several gut microorganisms are reported to have hypocholesterolemic activity (Nagpal 2012). The use of Bifidobacterium or Lactobacillus bacteria as hypocholesterolemic agents has been suggested by recent trials reporting that short-term administration of bacterial cells may reduce blood lipid levels in hypercholesterolemic subjects (Kumar 2012). However, it is not still clear if host diet differences might influence this phenotype. Furthermore, very limited information is available about the mechanism of cholesterol reduction of bifidobacteria.

In order to study in depth these potential abilities of bifidobacteria strains, our researches have focused on the cholesterol assimilation capacity in vitro of different bifidobacterial strains belonging to typical species of human gut. In addition, cholesterol depletion of the media by certain bifidobacterial strains was analysed.
We first noted that the level of cholesterol intake is highly variable between strains of the same species, this suggests that cholesterol intake capability is strain-specific feature rather than a species-related characteristic. In this context, in vitro tests involving *B. bifidum* PRL2010 cells show a considerable level of cholesterol assimilation. Notably, the transcriptome of *B. bifidum* PRL2010 cells grown in the presence of cholesterol reveal a specific induction of genes involved in transcription of ATP Binding Cassettes transporters. We may suppose that a specific mechanism for cholesterol intake exists in *B. bifidum* PRL2010, in order to insert cholesterol into the plasma membrane as was previously shown for other bacteria such as *Lactobacillus acidophilus* and *Lactobacillus plantarum* (Remagni 2013). Concurrently, cholesterol assimilation was shown to lead to alteration of membrane-specific fatty acid profiles.

The hypolipidemic potential of *B. bifidum* PRL2010 was assessed in two traditional animal models used in the atherosclerosis studies: the C57BL/6 mice and the ApoE knockout. Former are normolipidemic mice, whereas the latter mice are characterized by abnormally high levels of total cholesterol and low levels of HDL (Zhang 1992).

The administration of *B. bifidum* PRL2010 in the C57BL/6 mice have not revealed substantial differences of lipid profile among control and treated group, as well as no difference in mice weight. These data suggest that the administration of live microorganism in mice is harmless, evidenced by the maintenance of mice weight. Conversely, the administration of *B. bifidum* PRL2010 in ApoE knockout mice resulted in a slight decrease of total cholesterol. Despite the fact that statistical significance was not reached, which was probably related to the small sample size, this result is promising and should encourage further investigations. The use of this animal model has allowed to obtain more reliable results of the activity of *B. bifidum* PRL2010 in an animal model with abnormally high levels of total cholesterol and low levels of HDL.

In order to understand if probiotic administration has induced variations on circulating lipoprotein function, experiments of cholesterol efflux capacity of mice treated with probiotic or with placebo were performed. Lipoproteins are involved in a
physiological mechanism of Reverse Cholesterol Transport (RCT). RCT is the pathway whereby cholesterol is removed from peripheral tissues, including foam cells, delivered by HDL into blood and finally uptaken by liver for disposal into faeces (Glomset 1968). Results have revealed a slight increase of SR-BI mediated cholesterol efflux of cells incubated with sera of ApoE knockout mice treated with B. bifidum PRL2010. We may suppose that probiotic have induced a variation of lipoproteins profile, since the transporter SR-BI interacts exclusively with mature HDL. This variation of lipid profile is not confirmed by the ABCG1 efflux. Indeed the ABCG1 mediated efflux, which occurs with mature HDL, does not increase. Forward investigation may explain these data, using a 2D electrophoresis, in order to execute a qualitative analysis of plasma of treated mice.

According to the in vivo and in vitro results, several hypotheses on the mechanisms of hypolipidemic effects of B. bifidum PRL2010 can be proposed.

The bile salt hydrolase activity present in several gut bacteria, consists in the deconjugation of bile acids, resulting in reduced intestinal absorption and increased faecal elimination (Ooi 2010). The increase of BSH activity in Bb PRL2010, suggests that it may be a mechanism involved in cholesterol lowering capacity of this strain. Furthermore, blood cholesterol reduction may be associated with bacterial-mediated conversion to coprostanol of cholesterol uptaken into the bacterial cells (Lye 2010; Tahri 1997). Consistently, we observed an increased amount of this metabolite in cells enriched with cholesterol and micelles. This data are supported by transcriptomic analysis of bacterial genome, which has shown an up-regulation of genes encoding the reductase enzymes, when B. bifidum PRL2010 was grown in presence of cholesterol respect to PRL2010 cultivated in absence of cholesterol.

The reduction of circulating cholesterol may be due to another mechanism, as the cholesterol uptake into the bacterial cells. Indeed our data have shown that live and growing cells take up cholesterol more efficiently than dead cells. The majority of radiolabeled cholesterol was found in the cell envelope, whereas smaller amounts were associated with the bacterial cell wall hydrolysate. This data suggests that cholesterol is predominantly accumulated in the membrane, thus altering the membrane fatty acid composition. In addition, (as illustrated in figure 11), genes on
Bb PRL2010 encoding inorganic ion transporters and ATP-Binding Cassettes transporters are most significantly affected by the cultivation in the presence of cholesterol; conversely we did not observe significant variation for the functions of lipid metabolism and transport. This suggests that cholesterol is not triggering a catabolic response of *B. bifidum* PRL2010 to this compound (other than perhaps the conversion to coprostanol) and so the cholesterol lowering effect noticed for *Bb* PRL2010 cultures may be due to the intake of this sterol rather than its breakdown as previously noticed for other bacteria as mycobacteria (Van der Geize 2007).

Finally, the cholesterol-lowering activity exerted by *B. bifidum* PRL2010 cells may also be a consequence of the modulation of the gut microbiota provoked by this microorganism, resulting in a reduction of particular bacteria such as Collinsella spp. that positively correlate with total cholesterol (Lahti 2013). Overall, we report here for the first time the molecular response of bifidobacteria to cultivation in the presence of cholesterol and how this compound is assimilated by bifidobacterial cells, which ultimately was shown to result in a depletion of cholesterol from the environment.

**Figure 11:** Proposed mechanism of cholesterol lowering by *B. bifidum* PRL2010, a schematic representation of a cell and a metabolic internalization and conversion of cholesterol to coprostanol.
PART II:

Hypolipidemic Effects of a Nutraceutical Compound on Hamsters
Aim
Reverse Cholesterol Transport (RCT) is a physiological mechanism, which counteract the formation and development of atheroma, by promoting High Density Lipoproteins (HDL)-mediated removal of cholesterol from the artery wall, to be metabolized by liver into the faces as neutral sterols or bile acids (Rader 2009).

The analysis of patients’ lipoprotein profile is used to predict and to reduce cardiovascular diseases. The principal group of lipoproteins with atheroprotective function is the heterogeneous class of High Density Lipoprotein (HDL), which is lipoprotein differing in size, electrophoretic mobility and in lipid and protein composition (Rothblat 2010). Several epidemiological studies established that in humans the HDL cholesterol inversely correlates with the incidence of cardiovascular diseases (Menotti 2011). In addition, in the latest years it has been demonstrated that also the quality of HDL is important to define the atheroprotective activity of HDL (de la Llera Moya 2012). Indeed, the interactions between different subclasses of HDL with lipid transporters including ABC-transporter G1 and A1 (ABCG1/A1) and scavenger receptor BI (SR-BI) may differently modulate cholesterol efflux and the whole RCT. As a demonstration of this concept, it has been recently demonstrated that the measure of HDL capacity to promote cell cholesterol efflux is a predictor of cardiovascular risk in a wide range of subjects (Khera 2011). Even in animal models, the evaluation of plasma efflux potential may provide the mechanism for increased RCT (Zanotti 2008).

Consequently, pharmacological modulation of lipid profile in animals and humans is an important strategy to increase cholesterol efflux. As mentioned in literature, various potential drugs have been studied in depth, aimed to analyse their potential ability to increase the cholesterol efflux capacity, principally using animal models as mice and hamster (Christian 1999; Fullerton 2015; Briand 2010). Latter animal model has been used to assess diet-induced atherosclerosis since the early 1980s, because it presents high similarity to human cholesterol metabolism and enzymatic pathways compared with mouse model. Indeed advantages appeared to include a comparable receptor-mediated uptake of LDL cholesterol, cholesteryl ester transfer protein (CETP) activity, and uptake of LDL via the LDL receptor pathway (Dillard 2010).
The aim of this study is to analyse if the treatment of hamsters with a nutraceutical compound may modify the HDL functionality, in order to increase the cholesterol efflux from macrophages (first step of RCT).

This nutraceutical compound, which come from plants, is patent protected, and for practical reasons we call it “Ola”. Experimentations are part of an industrial project, which is not yet finalized.

In the last decades, nutraceutical field is spreading, in order to support the medical therapy or replace it, because they presents less side effects compared with drugs, and also they are more tolerated by patients.

Animal treatment was performed in the laboratory of prof. Jean Max Rouanet at the University of Montpellier 2, Montpellier, France, whereas the analysis of hamster’ sera capacity to promote cholesterol efflux, was performed in the pharmacological laboratories directed by prof. Franco Bernini, at University of Parma.

The treatment has consisted in a daily administration of four different amount of Ola via gavage for a 12 weeks in Golden Syrian Hamsters.

After animals’ sacrifice, the cholesterol efflux capacity of sera has been analysed, in order to examine the capacity of plasma of hamsters to stimulate the process involved in the first step of reverse cholesterol transport (RCT).
Materials and Methods
6.2.1 Animals and Treatment

Ninety male Golden Syrian hamsters with a middleweight of 85 g were bought from the society Janvier LABS (Le Genest-St-Isle, France).

After one week of acclimation period, they have been divided into 9 groups of 10 animals. They were housed in animal facility at University of Montpellier (France), subjected to a 12h/12h light/dark cycle at 23 ± 1°C and handled in compliance with European Union rules, and according to the “Guide for the Care and the Use of Laboratory Animals”, of National Institutes of Health Publication (NIH), published by the U.S. Government Printing Office, no.85-123(rev.) 1985, and the Committee for Animal Care of University Of Montpellier (France).

The first group (Standard group or STD) was fed with a standard diet whereas all other groups were fed with a High Fat diet (HF), as described below:

<table>
<thead>
<tr>
<th>Ingredients (g/kg)</th>
<th>STANDARD DIET</th>
<th>HIGH FAT DIET</th>
</tr>
</thead>
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<tr>
<td></td>
<td>g/kg</td>
<td>Kcal</td>
</tr>
<tr>
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</tr>
<tr>
<td>L-Methionin</td>
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</tr>
<tr>
<td>Maltodextrin</td>
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<td>120</td>
</tr>
<tr>
<td>Sucrose</td>
<td>290,5</td>
<td>1162</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>0</td>
</tr>
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<td>Vegetable Oil (corn/soy, 1/1)</td>
<td>45</td>
<td>405</td>
</tr>
<tr>
<td>Hydrogenated coconut oil</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>TOTAL</td>
<td>1000</td>
<td>3885</td>
</tr>
</tbody>
</table>

Table 3: Standard and high fat diet administered to hamsters during the treatment with Ola or the others drugs.
The different groups are summarized as follows:

- **Standard group (STD):** standard diet + 1mL tap water/day via gavage;
- **Control group (CTRL):** HF diet + 1mL tap water/day via gavage;
- **Atorvastatin group (Atorva):** HF atherogenic diet + atorvastatin 1.23 mg/kg/day, via gavage (corresponding to 10 mg/d in adult which is the recommended daily dose);
- **Lipanthyl group (LPT):** HF atherogenic diet + lipanthyl 17.88 mg/kg/day, via gavage (corresponding to 145 mg/d in adult which is the recommended daily dose);
- **Metformin group (MTF):** HF atherogenic diet + metformin 185 mg/kg/day, via gavage (corresponding to 1500 mg/d in adult which is the recommended daily dose);
- **Ola 1 group:** HF atherogenic diet + Ola dose 1, via gavage;
- **Ola 2 group:** HF atherogenic diet + Ola dose 2, via gavage;
- **Ola 3 group:** HF atherogenic diet + Ola dose 3, via gavage;
- **Ola 4 group:** HF atherogenic diet + Ola dose 4, via gavage.

All the Ola groups have increasing amount of “Ola” compound.

The experimental period of gavage was 12 weeks. At the end of this period, the hamsters were deprived of food overnight and fasting blood samples collected under lethal anaesthesia by cardiac puncture of pentobarbital.

### 6.2.2 Evaluation of Plasma Efflux Capacity

Plasma was isolated by low speed centrifugation at 2000g for 10 min and stored at -80°C. Before use, plasma was slowly defrosted in ice just before addition to cells as extracellular acceptors in cholesterol efflux experiments.
6.2.2.1 Measurement of passive diffusion and ABCA1-mediated cholesterol efflux

Efflux by passive diffusion and ABCA1 mediated efflux was measured using ShJ774 macrophage treated with cpt-cAMP to up-regulate ABCA1 and incubated with 1% (v/v) of hamster sera (Bortnick 2000). Method was described in chapter 5.2.7.1

6.2.2.2 Measurement of Cholesterol Efflux from Human Monocytic Cell Line Derived from an Acute Monocytic Leukaemia Patient (THP-1)

Efflux by human macrophages was measured using Human Monocytic Cell Line Derived from an Acute Monocytic Leukaemia Patient (THP-1) differentiated into macrophages incubating with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, U.S.A.) for 72 hours (Smith 1998). Cells were grown in Roswell Park Memorial Institute 1640 Medium (RPMI, Lonza, Switzerland) 10% Fetal Bovine Serum (FBS, Euroclone, Italy), incubated at 37°C in 5% CO2, seeded in 12-well plate and utilized at 80-90% confluence. Monolayers were radiolabeled with [3H]-cholesterol 2µCi/mL (Perkin Elmer, Italy) in medium containing 1% FCS. Following 24 hours labelling period, cells were washed and incubated with 0.2% of Bovine Serum Albumins (BSA, Sigma-Aldrich, U.S.A), with or without 5µg/ml of 22R-Hydroxycholesterol and 10µM 9-cis-retinoic acid (Sigma-Aldrich, U.S.A.), which are respectively agonist of Liver X Receptor (LXR), and Retinoid X Receptor (RXR). After 18 hours of incubation with these agonists, an up-regulation of transporters ABCA1 and G1 have occurred (Edwards 2002). 2 µg/ml of ACAT inhibitor (Sandoz 58035, Sigma-Aldrich, U.S.A.) was added during labelling and equilibration period to prevent cellular accumulation of cholesteryl ester (Zanotti 2012). In addition, in all steps was added 50ng/ml PMA. After equilibration period cells were incubated with 1% (v/v) plasma isolated from hamsters for 6 hours. The radioactivity in the medium was determined by liquid scintillation counting. Cholesterol efflux was calculated as a percentage of the radioactivity released into the medium over the radioactivity incorporated by cells before addition of plasma (Time zero). In order to analyse cellular [3H]-cholesterol content, cell monolayers
were extracted by the addition of 0.6 ml of 2-propanol (VWR International, U.S.A.). The lipid extracts were dried under a stream of N₂, re-suspended in toluene (VWR International, U.S.A.), and quantified by liquid scintillation counting. Every plasma sample was analysed in triplicate and the average and standard deviation have been obtained.

### 6.2.3 Statistical Analysis

Values are expressed as mean ± SD. Statistical analyses were performed with Prism 5 software (GraphPad Software, San Diego, California). Statistically significant differences among the means of different groups were tested using analysis of variance (ANOVA one way). The Bonferroni test was performed after ANOVA and p value of <0.05 was considered significant.
Results
### 6.3.1 Lipid Profile of Hamsters after Treatment with Ola or Drugs

Table 4 shows the lipid profile of hamsters after 12 weeks of treatment. The values of HDL, total cholesterol, LDL, and triglycerides are expressed as mean of value of hamsters composing each group described above.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>3,30</td>
<td>0,14</td>
<td>2,47</td>
<td>0,01</td>
<td>0,83</td>
<td>0,14</td>
<td>1,31</td>
<td>0,21</td>
</tr>
<tr>
<td>Control</td>
<td>10,76</td>
<td>1,77</td>
<td>3,57</td>
<td>0,48</td>
<td>7,19</td>
<td>2,09</td>
<td>2,63</td>
<td>1,02</td>
</tr>
<tr>
<td>Atorva</td>
<td>15,34</td>
<td>0,89</td>
<td>4,53</td>
<td>0,80</td>
<td>10,81</td>
<td>1,39</td>
<td>0,68</td>
<td>0,12</td>
</tr>
<tr>
<td>Lipantil</td>
<td>10,33</td>
<td>0,88</td>
<td>3,80</td>
<td>0,47</td>
<td>6,53</td>
<td>1,12</td>
<td>2,77</td>
<td>0,86</td>
</tr>
<tr>
<td>Metformin</td>
<td>6,91</td>
<td>0,66</td>
<td>2,95</td>
<td>0,30</td>
<td>3,97</td>
<td>0,95</td>
<td>1,49</td>
<td>0,09</td>
</tr>
<tr>
<td>Ola 1</td>
<td>10,25</td>
<td>0,81</td>
<td>3,93</td>
<td>0,44</td>
<td>6,32</td>
<td>1,15</td>
<td>1,27</td>
<td>0,61</td>
</tr>
<tr>
<td>Ola 2</td>
<td>8,90</td>
<td>0,81</td>
<td>4,14</td>
<td>0,37</td>
<td>4,76</td>
<td>0,75</td>
<td>1,38</td>
<td>0,51</td>
</tr>
<tr>
<td>Ola 3</td>
<td>8,29</td>
<td>1,09</td>
<td>3,86</td>
<td>0,44</td>
<td>4,42</td>
<td>1,22</td>
<td>1,33</td>
<td>0,68</td>
</tr>
<tr>
<td>Ola 4</td>
<td>9,22</td>
<td>1,28</td>
<td>3,67</td>
<td>0,31</td>
<td>5,55</td>
<td>1,20</td>
<td>1,22</td>
<td>0,33</td>
</tr>
</tbody>
</table>

Table 4: Lipid profile of hamsters after administration of Ola or drugs for 12 weeks via gavage.
6.3.2 Evaluation of Plasma Efflux Capacity of Hamsters Treated with Ola

Cholesterol efflux from cells is the rate limiting step of macrophage RCT, as it regulates the amount of cholesterol released from foam cells of the arterial wall and available to be delivered to the liver for disposal (Rader 2009). In order to investigate if the administration of the nutraceutical compound “Ola” (not real name, because under patent) may influence cholesterol efflux from foam cells of atherosclerotic plaque, experiments have been performed using the plasma of hamsters treated with or without Ola on THP-1 which are human macrophages and on murine macrophages J774 that express the transporter ABCA1 (Bortnick 2000).

6.3.2.1 Cholesterol Efflux from Human Macrophage THP-1

![Graph 22](image)

**Graph 22**: This graph shows the efflux from THP-1 cells of plasma of hamsters treated with Ola, or with all other drugs. THP-1 monocytes, after differentiation in macrophages, were labeled with 1 μCi/mL [³H]-cholesterol in RPMI 1640 with 1% FCS for 24 hours. Cells were washed and incubated with 0.2% of Bovine Serum Albumins (BSA), with or without 5µg/ml 22R-hydroxycholesterol and 10µM 9-cis-retinoic acid for 18h. Both molecules are agonist of Liver X Receptor (LXR), which induces an up-regulation of transporters ABCA1 and G1. Then, cells were incubated for 6 h with 1% plasma isolated from hamsters treated with or without Ola. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0x100± SD. Statistical analysis were performed using Anova One way, with Dunnett’s Multiple Comparison test, comparing control group with all others groups. ** vs control group.
As shown in the graph 22, there are not substantial differences of thp-1 efflux among control group and groups of hamsters treated with Ola dose 1 and 2. Conversely we observed a slight, but not significant reduction of cholesterol efflux of cells treated with Ola dose 3 and 4, compared with control group. Significant efflux increase has been observed among control and standard group (p<0.001).

### 6.3.2.2 Cholesterol Efflux from Murine Macrophages J774

- **ABCA1 Mediated Efflux from J774 Cells**

As graph 23 shows, a slight increase of ABCA1 mediated efflux in J774 cells of Ola 4 group compared with control group has been observed. In addition, a slight increase of cholesterol efflux of control group compared with standard group has been observed. This difference do not reach significantly value, but, they tend to significance (p values respectively p=0.10).

**Graph 23:** This graphic shows the efflux ABCA1 mediated of plasma of hamsters treated with Ola, or with all other drugs. J774 macrophages were radiolabeled with [3H]-cholesterol for 24 hours, in DMEM medium with 1% FCS and 2µg/mL of an ACAT inhibitor. Then, cells were incubated for 18h with 0.2% BSA in presence of 0.3mM cpt-cAMP, washed, and incubated for 4h with 1% (v/v) plasma isolated from hamsters treated with Ola or the other drugs. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0 x100 ± SD. Values of ABCA1 mediated efflux was obtained as difference among total efflux minus passive diffusion. Statistical analysis were performed using Anova One way, with Dunnett’s Multiple Comparison test, comparing control group with all others groups.
- **Total Efflux from J774 Cells**

The total efflux of cells incubated with plasma of hamsters treated with the Ola 1 and 2 is significantly increased, showing a p value <0.05. The Ola groups 3 and 4 presents a slight increase compared with control group, with a p value respectively of 0.0621 and 0.0552 (graph 24).

![Graph 24](image_url)

**Graph 24**: This graphic shows the total efflux from J774 cells of plasma of hamsters treated with Ola, or with all other drugs. J774 macrophages were radiolabeled with $[^3]$H-cholesterol for 24 hours, in DMEM medium with 1% FCS and 2µg/mL of an ACAT inhibitor. Then, cells were incubated for 18h with 0.2% BSA in presence of 0.3mM cpt-cAMP, washed, and incubated for 4h with 1% (v/v) plasma isolated from hamsters treated with Ola or the other drugs. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0 x100± SD. Statistical analysis were performed using Anova One way, with Dunnett’s Multiple Comparison test, comparing control group with all others groups. * vs control group.

As graph 25 show, passive diffusion of atorvastatin group resulted significant increased (p<0.01) compared with control group. Whereas plasma of hamsters treated with metformin and Ola 4 have slightly increased the passive diffusion of J774 cells.
- Passive Diffusion of J774 Cells

Graph 25: This graphic shows the total efflux from J774 cells of plasma of hamsters treated with Ola, or with all other drugs. J774 macrophages were radiolabeled with \(^{3}H\)-cholesterol for 24 hours, in DMEM medium with 1% FCS and 2µg/mL of an ACAT inhibitor. Then, cells were incubated for 18h with 0.2% BSA in presence of 0.3mM cpt-cAMP, washed, and incubated for 4h with 1% plasma isolated from hamsters treated with Ola or the other drugs. The experiment was performed in triplicate. Efflux was expressed as counts per minute in medium/counts per minute of time 0 x100± SD. Statistical analysis were performed using Anova One way, with Dunnett’s Multiple Comparison test, comparing control group with all others groups. ** vs control group.
6.3.2.3 Correlations among Lipid Profile and Cholesterol Efflux

Graph 26: These graphs show the significantly correlation among triglycerides and ABCA1 mediated efflux, or total efflux or passive diffusion of J774 cells after incubation with plasma of hamsters treated with Ola 2 (left side) and Ola 4 (right side). Data were analysed by linear regression.
As panel 26 shows, a significantly correlation (p< 0,05) among ABCA1 mediated efflux and triglycerides has been found. Former graph shows that increasing amounts of triglycerides present in the plasma of hamsters after administration of Ola 2 are associated with an increase of cholesterol efflux in J774 cells.

Total efflux of J774 cells was increased after incubation with plasma of hamsters treated with Ola 2 and 4. In particular total efflux presents a high correlation with triglycerides after administration of Ola 2 and 4 as well, respectively with a p value < 0,05 and 0,01. A correlation among total cholesterol and total efflux of J774 cells was found after exposition to plasma of hamsters treated with Ola 4 (p value < 0,05) (graph 27).

In addition, both graphs show a correlation among passive diffusion of J774 cells and triglycerides fraction of plasma of hamsters treated whit Ola 2 and 4. A positive correlation (p<0,05) was found between passive diffusion and triglycerides after administration of Ola 2, whereas a reduction of passive diffusion depending by amount of triglycerides has been observed when J774 cells were incubated with hamster’s plasma of the Ola 4 group.
Discussion
Reverse Cholesterol Transport (RCT) is a physiological mechanism, which counteract the formation and development of atherosclerotic plaque, by promoting the activity of High Density Lipoproteins (HDL). These lipoproteins are responsible of the cholesterol removal from foam cells of atheroma, and the following metabolism in the hepatic cells, in order to excrete the cholesterol into the faeces as neutral sterols or bile acids (Rader 2009).

Several epidemiological studies established that the HDL cholesterol inversely correlates with the incidence of cardiovascular diseases (Menotti 2011). The heterogeneous population of HDL is the principal group of lipoproteins involved in the cholesterol efflux (Rothblat 2010). Indeed, the interactions between different subclasses of HDL with ABCG1, ABCA1, and SR-BI may differently modulate cholesterol efflux and the whole RCT. As a demonstration of this concept, several studies have demonstrated that the HDL capacity to promote cell cholesterol efflux is a predictor of cardiovascular risk in humans (Khera 2011). Consequently, pharmacological modulation of lipid profile in animals and humans is an important strategy to increase cholesterol efflux.

The modulation capabilities on cholesterol efflux of nutraceutical compound “Ola” were tested in the hamster model, because it has high similarity to human cholesterol metabolism and enzymatic pathways compared with mouse model. Indeed advantages appeared to include a comparable receptor-mediated uptake of LDL cholesterol, cholesteryl ester transfer protein (CETP) activity, and uptake of LDL via the LDL receptor pathway (Dillard 2010).

Cholesterol efflux from cells is the rate limiting step of macrophage RCT, as it regulates the amount of cholesterol released from foam cells of the arterial wall and available to be delivered to the liver for disposal (Rader 2009). In order to investigate if the administration the nutraceutical compound “Ola” (not real name, because under patent) influence cholesterol efflux from foam cells of atherosclerotic plaque, experiments have been performed using the plasma of hamsters treated with or without Ola on THP-1 which are human macrophages and on murine macrophages J774 that express the transporter ABCA1 (Bortnick 2000). ABCA1 is unidirectional ATP-depending transporter that carries out cholesterol against concentration gradient. It interacts with lipid free ApoA1 generating discoidal HDL particles (prech-
HDL) (Tall 2008). We decided to focus of ABCA1 mediated efflux because the contribution of this transporter is predominant. Adorni et al. demonstrated that in murine peritoneal macrophages, ABCA1 contribution is predominant and represents 35% of total efflux, ABCG1 contributes less than ABCA1 (21%); while SR-BI contribution is minimal (Adorni 2007). As Larrede et al demonstrated, these relations are maintained in humans, in particular ABCA1 still remains the predominant contributor to cholesterol efflux in cholesterol loaded human macrophages but, differently from mice, SR-BI is relatively more important than ABCG1 (Larrede 2009).

The four doses of Ola did not influence significantly the cholesterol efflux on THP-1 macrophages. Conversely the incubation of plasma of the four Ola groups with J774 cells led to obtain interesting results. Indeed, compared with control groups, the total efflux, which is the sum of passive diffusion and ABCA1 mediated efflux, is increased. In particular, the Ola with the lowest dose (Ola 1) has significantly increased the total efflux of J774 cells. This may be explained in part by the slight increase of ABCA1 mediated efflux observed, which mainly contribute to cholesterol efflux from J774 cells (Fournier 2012). Also the Ola 2 group presents a significantly increase of total efflux. In addition, Ola 2 group presents a significant correlations among the values of triglycerides, and percentage of passive diffusion, total efflux, and ABCA1 mediated efflux.

In addition, the incubation of plasma of animals treated with Ola 4 with J774 cells induces a slight increase of total and ABCA1 mediated efflux. Furthermore, also in Ola 4 group, significant correlations has been found, among the percentage of total efflux, ABCA1 mediated efflux and passive diffusion with values of total cholesterol and triglycerides.

These correlations demonstrate that the Ola 2 and 4 lead to a modulation of lipid profile of hamsters, influencing the cholesterol efflux capabilities of triglycerides enriched lipoproteins of hamsters after treatment with the Ola. As literature suggested, a positive correlation among triglycerides and ABCA1 transporters of macrophages of foam cells of atherosclerotic plaque exists. This correlation results in an increase of cholesterol efflux (Weibel 2014).
The positive activity of the nutraceutical compound “Ola” on cholesterol efflux might be a strategy against the atherosclerosis, increasing the cholesterol efflux capacity of “foam cells” situated in atheroma. Our results have shown as the administration of a nutraceutical compound may be a potential therapeutic strategy in order to reduce cholesterol levels in hypercholesterolemic patients, supporting pharmacological drugs or in some case, replace them.
Bibliography


Hennequin, C, C Kauffmann & Infec.


Larrede, Sandra, Carmel M Quinn, Wendy Jessup,


Sozzi, T. 2006. “Method for Preventing the Inactivation, due to Specific Bacteriophages, of Probiotic Strain Mixtures Used in Cattle-Breeding.”

Spinosa, Maria Rita. 2000. “In a French Hospital Caused by a Strain Related to an Italian Probiotic?” *Microbial Ecology in Health and Disease* 12 (Table 1): 99–101.


Tietge, Uwe J F, and Albert K Groen. 2013. “Role the TICE?: Advancing the Concept of Transintestinal Cholesterol Excretion.” *Arteriosclerosis, Thrombosis, and Vascular Biology*.


Ringraziamenti/Acknowledgements

E quindi siamo arrivati ai ringraziamenti... per la seconda volta....chi lo avrebbe mai detto!!

Dal primo giorno della scuola dell’Infanzia fino ad oggi so che siete sempre stati là ad aiutarmi a crescere e diventare quello che sono adesso .... E so che tutto questo lo devo a voi ... grazie Mamma e Papà per tutto quello che avete fatto per me, ed è a voi che dedico la mia tesi. Grazie alle mie tre sorelle: Cinzia, Rosa Anna, Chiara per abbellire le mie giornate con casini e risate, che ti fanno sempre sentire a casa, ovunque noi ci troviamo, dispersi per il mondo.

Un doveroso ringraziamento lo devo al Prof. Bernini, per avermi permesso di trascorrere questi anni all’interno del suo gruppo di ricerca e ancor di più per avermi aiutato a corredare questa esperienza con i vari periodi di formazione all’estero. Inoltre grazie a tutto il laboratorio del Corpo C, Lato Sud del dipartimento di Farmacia, che mi ha aiutato a crescere, partendo da un ingenuo tesista fino a diventare un ingenuo dottorando, che ogni giorno ha affrontato tutte le novità belle o brutte che ci attendevano dietro la porta d’ingresso del labo (dalle cellule che non andavano, agli ordini, ai risultati, e tante altre cose).

Grazie ad Ilaria per la pazienza e per la fiducia, nonostante i vari sbagli e le dimenticanze sempre dietro l’angolo, grazie per avermi coinvolto in tutti i lavori e trasmesso la voglia di lavorare, di approfondire quello che stavo facendo e soprattutto per il senso del dovere... grazie per gli insegnamenti, per avermi fatto capire come si ragiona su un esperimento, e di non essere impulsivo davanti a dati che magari non sono quelli che ci aspettavamo. Grazie per aver corretto le mille versioni di un abstract o delle tesi, e di aver ascoltato tutte le mie teorie, molte volte “stramalate”, su avvenimenti scientifici. Grazie di tutto.....

E adesso mi tocca anche ringraziare una ragazza (P.h.D., ndr) che mi ha accompagnato dal primo giorno in labo fino ad adesso. Quando avevo un dubbio, un problema o anche una mia paranoia lei c’era sempre lì ad ascoltarmi e consigliarmi.... E pensare che dopo la prima cazziata che mi hai fatto quando ero tesista, non vedeva l’ora che te ne andassi in Olanda, così sparivi per un bel pò.... E adesso invece dirti Grazie è troppo poco, grazie per le risate, per le confessioni, per gli scherzi fatti e ricevuti, per l’amicizia che si è creata... (anche se rimani sempre la “dottoranda cattiva” 😊)... Grazie Daniela.

E poi tocca all’altro pezzo della coppia (che amava prendermi in giro), la sempre calma Giulia, che mi ha accompagnato nelle mille ristate e avventure della tesi e dell’inizio del dottorato... grazie per tutti gli insegnamenti sotto cappa, per aver risposto ad ogni mia stupida domanda e per tutti i momenti felici...
Simo di te non parlo, che è meglio... ti dico solo che per nostra fortuna non abbiamo iniziato il dottorato insieme, senzò non avremmo concluso nulla, ricordati però una cosa, la più importante: forza Juve!! Come posso non parlare delle nuove dottorande Ele e Laura, che sopportano me e Simone, e sentono tutte le nostre auliche discussioni su importanti temi di attualità. Grazie per le fasi di panico e scasso (Eleonora, ndr) e per il pieno di pensieri negativi (Laura, ndr), ma soprattutto per i bei momenti passati in labo insieme, per le risate e per la vostra pazienza nel chiarire ogni mio dubbio e aiutarmi....

Grazie ad Elda per le risate a pranzo e durante le giornate pesanti in cui un sorriso serviva sempre. E alla neo mamma (auguri ancora) Mapi, è stato bello lavorare con te, grazie a Nicoletta e a tutta la sua allegria e soprattutto i suoi dolci. Un grazie anche a Francesca, sempre pronta ad aiutarmi ogniqualvolta chiedeva dei chiarimenti, e grazie soprattutto per la tua compagnia nel periodo di elaborazione della tesi... durante il quale tra una risata e una risposta al telefono: «No, Francesca non c’è, ti faccio richiamare!» Ho passato un bel periodo.

Grazie a tutti, le tesiste ed i tesisti, che in questi anni si sono susseguiti e a cui ho trasmesso quel poco che so, e che mi hanno aiutato nei miei esperimenti e non solo. Grazie soprattutto a Carlotta, Cinzia, Camilla, Vale, Gemma e l’unico tesista maschio, Giampy per tutte le giornate passate in labo, tra cellule e topi da sacrificare, anche se sentire Adele di prima mattina non è il massimo.

Il ringraziamento più bello ed importante va ad una tesista in particolare (ex tesista purtroppo, ndr). una tesista che è entrata silenziosamente in labo.... E così, senza accorgermene è entrata nei miei pensieri, nei miei gesti, nella mia vita.... Ed è sicuramente la più bella scoperta che abbia mai potuto fare (che a confronto, quelle su Nature e The Lancet, sono nulla). Grazie a te, ho scoperto come stare bene insieme ad una persona, ho scoperto come condividere la quotidianità, le sconfitte e le vittorie di ogni giorno. Grazie a te ho scoperto come essere sereno e felice se si fa di tutto per rendere felice e serena l’altra persona. Ho imparato a prendere l’aereo come se fosse un bus (avevo paura dell’aereo), e a fare tanti km solo per vederti... ma ne è sempre valsa la pena. Ne avremo di chilometri da fare insieme in futuro... grazie di tutto Enrica, per avermi sempre sostenuto, soprattutto in quest’ultimo periodo abbastanza complicato, e per tutti i viaggi e i Nostri momenti.. ....

E poi un grazie agli amici di una vita, che sono sempre li, anche se non ci vediamo tutti i giorni, a sostenerci, a scherzare, a fare serate ignoranti insieme... a loro non interessa un grazie, interessa l’alcool che dovrò offrire loro per questo traguardo che grazie anche a loro, sono riuscito a raggiungere.
Piero e Pasquale, a voi dire grazie e quasi superfluo...non solo per il dottorato, ma per tutti gli anni passati insieme a fare di tutto, dalla serata ignorante alla discussione seria...so che ci siete sempre stati e sempre ci sarete...

Grazie alla famiglia di Parma, che ha accompagnato in tutti questi anni le mie avventure in questa città che con loro si è trasformato in qualcosa di speciale....una seconda casa.... Carmine, Enzo, Angela, Antonella, Robby, Egidio (gino), Danilo (nylon), Flora.

E poi grazie a tutta la gentaglia di Monte Sant’Angelo. Ogni volta che scendo giù c’è sempre un motivo per una festa.... Marco e tutte le sue polemiche, Salvo e tutti i suoi pisciatuiri (Apple, ndr), Michela, Biaggino il chimico, Marilina (poropiropò), Cristian, Viviana, Dodo, Giuseppe (Cota) e Mariangela. E poi ai montanari trasferiti al Tofee bar a Parma (Marzia), con cui abbiamo preso residenza li, Il Duca Flavio Pio Renzulli e il piccolo Pasqualone.

Grazie alla famiglia tutta... dagli zii ai cugini... è sempre bello stare con voi, e con il casinò che si crea.... in Particolare a Zio Tonino... grazie per tutti questi anni di appoggio qui a Parma e per tutti i pranzi della domenica.. 😊

Grazie ad Antonio Grande, il Cugino, che mi ha sempre dato consigli, e ottimo confessionale.... In bocca al lupo per il grande passo con Enza ... 😊 grazie anche all’altro Antonio, il Coccia e alla sua bella famiglia che si sta allargando... (Auguri Vale!!). Un ringraziamento particolare a nonno Salvatore, nonna Rosa, nonno Antonio e nonna Vincenza, che sono sempre con noi... che sono sicuramente contenti di aver creato una bella e grande famiglia... 😊

Acknowledgement: French Part

A sincere special thanks to Prof. J.M Rouanet and Sylvie, first of all for accepting me in your research group in Montepellier, and also for all your teaching about how manage the hamsters. Thanks to Julienn for the availability and for the collaboration. And finally. Thanks to Cindy and Jorys for all the for your essential help to realize this work and for your scientific and personal support.. thanks for all the time spent with me in the laboratory and outside.... 😊

Acknowledgement: Switzerland Part

A sincere special thanks to MD Prof. Arnold von Eckardstein and Lucia Rohrer for accepting me in your research group in the Institut für Klinische Chemie of UniversitätsSpital of Zürich.
A special thanks to Damir a Silvija for your availability to clarify all my doubts especially in the first part of my Zurich experience …. Thanks a lot for all the laughs and all the coffee break!!

Paolo grazie di tutto, senza te tutto sarebbe stato più complicato / thanks for all the funny moments occurred in the lab, for the support, during the “wisdom time and in the brain storming time”. Also, for all the advices for my future and how to live in Zurich, and especially for the “cultural moments” of our discussion during the coffee break, or over a lot of beers. Thanks to Reda, for the laughs and the discussion about the Power (remember, Reda, remember) 😊.

Finally I would say thanks to a great researcher and friend, with which I collaborated: Vidya. Thanks for your patience whit me, for all your teaching and for you excellent Indian cuisine, so good !! I hope in the future to work with you. 😊 to conclude… thanks all people which work in Wagistrasse 14, Zurich.